INVESTIGATIONS OF THE THROMBIN GENERATION TEST FOR THE MEASUREMENT OF FACTOR VIII

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ABSTRACT

Haemophilia A is a genetic bleeding disorder in which the plasma level of the clotting protein FVIII is reduced or absent. Treatment of haemophilia is by replacement of the missing FVIII with FVIII concentrates made from human plasma, or by recombinant technology. The two widely used assays for FVIII measurement (one-stage APTT and chromogenic assay) have disagreements in potency of FVIII concentrates and in post-infusion plasma samples; these discrepancies are largest for the recombinant products. The aim of this project was to use a modified thrombin generation test (TGT) for a more physiological approach to FVIII measurement.

Two different TGTs were used, a clotting based and a fluorogenic method. Thrombin generation was carried out in commercial FVIII deficient plasma to which a FVIII concentrate was added, the reaction was triggered with the addition of phospholipid, calcium and FIXa. A range of FVIII concentrates were investigated, four plasma-derived, three recombinant products (two full-length, one B-domain deleted). There were no substantial differences between the concentrates under the various conditions tested. Platelets were added to the system to investigate if the different amounts of von Willebrand factor (VWF) in the concentrates affected the thrombin generation, but no substantial differences were observed between low purity plasma-derived and recombinant products containing no VWF.

It was also found that a large amount of thrombin could be generated at low levels of FVIII, and both assays were sensitive to FVIII levels of 0.001 IU/ml. However, it was dependent upon the high amount of FIXa used to trigger the reaction. A wide variation in the amount of thrombin generated by several commercial FVIII deficient plasmas was also observed, both between different manufacturers, and between batches of the same plasma. The ability to detect low levels of FVIII could be beneficial for monitoring patients during prophylaxis, gene therapy and in phenotyping patients with severe haemophilia.

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ABBREVIATIONS

Ab	Antibody
ACD-A	Acid citrated dextrose-A
ADP	Adenosine diphosphate
APC	Activated protein C
APTT	Activated partial thrombin time
AT	Antithrombin
AUC	Area under the curve
BDD	B-domain deleted
BSA	Bovine serum albumin
CHO	Chinese hamster ovary
CRM	Cross reactive material
CV	Coefficient of variation
DDAVP	1-deamino-8-D-arginine vasopressin
DIC	Disseminated intravascular coagulation
DNA	Deoxribose nucleic acid
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ERGIC	ER-Golgi intermediate compartment
ETP	Endogenous thrombin potential
FII	Prothrombin
FIIa	Thrombin
FIX	Factor IX
FIXa	Activated FIX
FV	Factor V
FVa	Activated FV
FVII	Factor VII
FVIIa	activated FVII
FVIII	Factor VIII
FVIIIa	Activated FVIII
FX	Factor X
FXa	Activated FX
FXI	Factor XI
FXIa	Activated FXI
FXIII	Factor XIII
FXIIIa	Activated FXIII
GBS	Glycine buffered saline
GP	Glycoprotein
HP	High purity
HSA	Human serum albumin
HSPG	Heparan sulfate proteoglycan
IP	Intermediate purity
IS	International standard
ISTH	International Society of Thrombosis and Haemostasis
IU	International unit

LRP	Lipoprotein receptor-related protein
mRNA	messenger RNA
NIBSC	National Institute for Biological Standards and Controls
NP	Normal plasma
OD	Optical density
OHP	Overall haemostatic potential
OPD	Orthophenylenediamine
PAR	Protease activated receptor
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PD	Plasma derived
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PL	Phospholipid
PRP	Platelet rich plasma
PS	Phosphatidylserine
PT	Prothrombin time
RAP	Receptor associated protein
RFU	Relative fluorescent units
RNA	Ribose nucleic acid
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
rFVIIa	Recombinant FVIIa
SPH	Sphingomyelin
SSC	Scientific and Standardisation Committee
$T_{½max}$	Time to reach half peak-height
TAFI	Thrombin activated fibrinolysis inhibitor
TDT	Thrombin dynamics test
TEG	Thromboelastogram
TF	Tissue factor
TFPI	Tissue factore pathway inhibitor
TGT	Thrombin generation test
tPA	Tissue type plasminogen activator
uPA	Urinary type plasminogen activator
VWD	von Willebrand disease
VWF	von Willebrand factor

CHAPTER 1
INTRODUCTION

1.1 Haemostasis.

1.1.1 Overview

Haemostasis is a physiological process by which a clot is formed after injury to a blood vessel to prevent blood loss. Haemostasis can also occur in pathological states in which it is termed thrombosis.

Injury to the vessels causes an initial vasoconstriction which helps to slow the blood flow. The damaged endothelium of the vessel exposes the subendothelial tissue which attracts platelets and causes them to adhere to the damaged area to form the primary haemostatic plug. The adhesion of the platelets cause them to release soluble factors such as von Willebrand factor (VWF), which, together with tissue factor (TF) initiates the coagulation pathway. This involves the activation of a series of proteases in sequence. Each protease activates the next protease in the pathway leading to an escalating response which culminates in the production of a stable fibrin clot from fibrinogen by the protease thrombin.

1.1.2 Clotting cascade

Coagulation is a series of reactions in which one serine protease activates the next one in the series. This was likened to a waterfall or cascade of reactions (Davie & Ratnoff, 1964;Macfarlane, 1964) and allows amplification of the process.

Coagulation was originally thought to follow two different paths, the "extrinsic" which is caused by tissue factor (TF) activation and "intrinsic" which is contact activation by a negatively charged surface such as glass (*in vitro*) or collagen (*in vivo*) (Fig 1.1). However, it is now known that there are many positive and negative feedback loops to the system and it is not quite as straightforward as was initially envisaged (Fig 1.2). The intrinsic pathway causes Factor XII (FXII) to become activated FXII (FXIIa). FXIIa in turn activates FXI,

which in turn activates FIX. The extrinsic pathway is initiated by TF which forms a complex with FVII or FVIIa. Both FIXa, with cofactor FVIIIa, and TF-VIIa are able to activate FX on a phospholipid (PL) surface and the subsequent steps of coagulation are termed the common pathway. FXa activates prothrombin to thrombin, also in the presence of the cofactor FVa and PL. Thrombin is the crucial component of coagulation cascade, causing back activation of coagulation factors higher up in the pathway, allowing more thrombin to be generated, and importantly activating fibrinogen to fibrin which allows a clot to form.

These extrinsic and intrinsic definitions are still used in laboratory diagnosis. Prolongation of the prothrombin time (PT) reflects a deficiency in the extrinsic pathway, whereas a prolongation in the activated partial thromoboin time (APTT) would suggest a deficiency in one of the components of the intrinsic pathway. A prolongation in both suggests a deficiency in the common pathway.

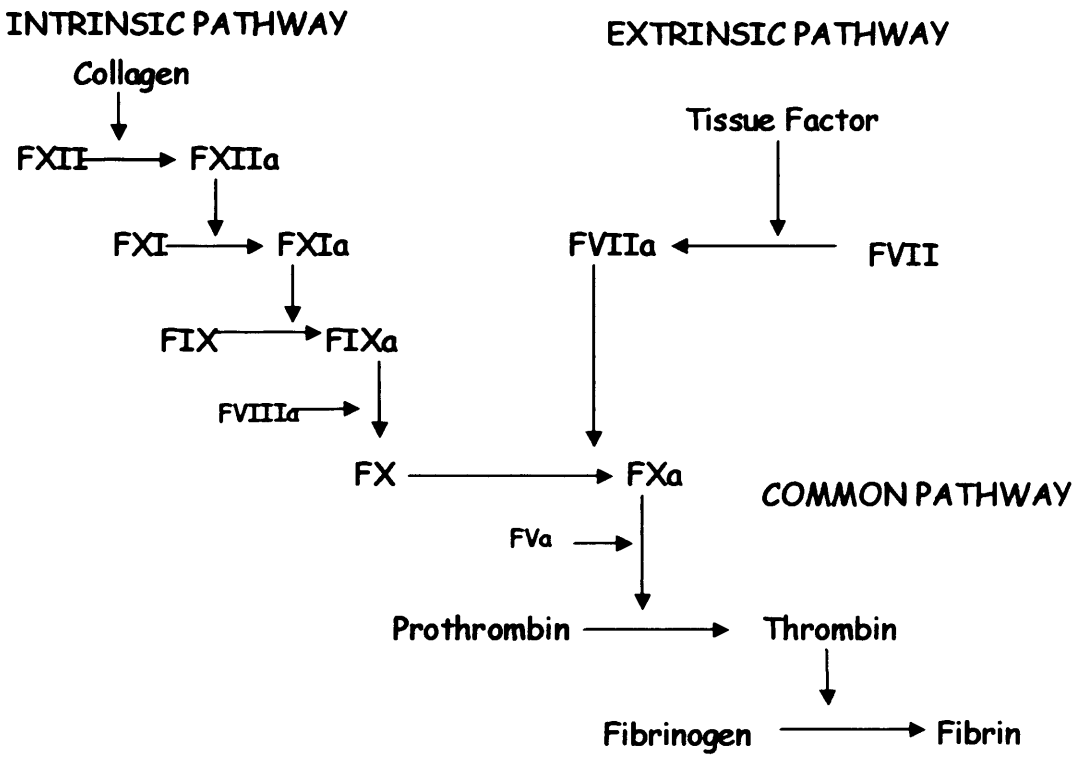


Fig 1.1 Intrinsic and extrinsic pathways of coagulation

There are however, limitations with these definitions as deficiency of FXII despite causing a prolongation of the APTT does not result in a bleeding problem, whereas deficiencies in FVIII and FIX can cause severe bleeding tendencies and are termed haemophilia A and B. The study of a patient whom was FVII deficient led to the conclusion that *in vivo* haemostasis proceeded via the extrinsic system (Josso & Prou-Wartelle, 1965).

1.1.2.1 Initiation

TF, which is exposed following vessel damage is thought to be the main initiator of coagulation *in vivo* (Rapaport & Rao, 1995). TF is a transmembrane glycoprotein which comprises of three regions, the transmembrane domain, a cytoplasmic tail and an extracellular domain which is required for haemostatic activity. For TF to be active in haemostasis, lipid binding is required (Paborsky *et al*, 1991). TF was not thought to circulate in the blood due to the lipid binding requirements, there is now evidence though that TF is contained on microparticles which are small fragments of cell membranes predominantly derived from platelets (Berckmans *et al*, 2001).

FVIIa is an inefficient enzyme to activate FX, however when TF is in complex with FVIIa the activity increases by over a millionfold (Morrissey, 2001). The initial TF-VIIa complex is formed with small amounts of FVIIa which circulates under normal conditions. FVII in complex with TF can undergo autoactivation to form TF-FVIIa (Nakagaki *et al*, 1991), which is an additional mechanism by which sufficient TF-VIIa complex can be formed. The trace amounts of activated FVIIa-TF are held in check by tissue factor pathway inhibitor (TFPI) (van't Veer & Mann, 1997).

Under basal conditions one percent of total FVII circulates as FVIIa (Wildgoose *et al*, 1992). It is thought that FVIIa which circulates during basal conditions arises through activation by FIX or FIXa as patients deficient in FIX have reduced levels (Wildgoose *et al*, 1992), which can be increased following FIX infusion (Eichinger *et al*, 1995). In addition small amounts of activated FIX and FX also circulate under normal basal condition, these

are markedly reduced in FVII deficient patients (Bauer *et al*, 1992), which would suggest that TF-VIIa pathway is responsible for the generation of FIXa and FXa under normal basal conditions.

1.1.2.2 Tenase complex

The circulating TF-FVIIa forms a complex with Ca^{2+} and a source of PL, such as cell surfaces or activated platelets, this complex is able to activate FX to FXa in the "extrinsic tenase" complex (Williams, 1964;Radcliffe & Barton, 1973;Jesty & Silverberg, 1979;Baugh *et al*, 2000). However, the "extrinsic tenase" complex does not generate the majority of the FXa, this is generated by the "intrinsic tenase" in which FIXa activates FX on a phospholipid surface with FVIIIa acting as a cofactor for this reaction, which increases the catalytic efficiency of the complex by eight-fold (McGee & Li, 1991).

FIX can be activated by two mechanisms, the first to occur in coagulation is by the TF-VIIa complex (Østerud & Rapaport, 1977;Lawson & Mann, 1991;Bauer *et al*, 1990) and the second is FXIa. It is thought that activation by TF-VIIa is more rapid than FXIa (Bajaj *et al*, 1983). Before FXIa can activate FIX, it must be activated by thrombin, and therefore sufficient thrombin must be generated first (Naito & Fujikawa, 1991). This pathway is important when low TF concentrations initiate haemostasis (Keularts *et al*, 2001a;von dem Borne *et al*, 1995) and for thrombin generation as activation by TF-FVIIa is short lived owing to inhibition by TFPI.

During the initiation phase FXa is solely generated by FVIIa-TF, whereas initial thrombin (FIIa) is generated by FVa-Xa or FV-Xa. Once a small amount of thrombin has been generated, it can activate FVIII, FV, FVII and FXI leading to a burst and amplification of thrombin generation (Butenas *et al*, 1997).

1.1.2.3 Prothrombinase complex

Prothrombin is activated to form thrombin by the action of FXa and in the presence of cofactor FVa, $Ca^{2+}$and on a phospholipid surface. Initially FV is able to act as a cofactor in the prothrombinase complex, although it is 100-fold less effective than FVa (Foster *et al*,

1983). Once a small amount of thrombin has been generated, thrombin can activate FV to FVa (Giddings & Bloom, 1975).

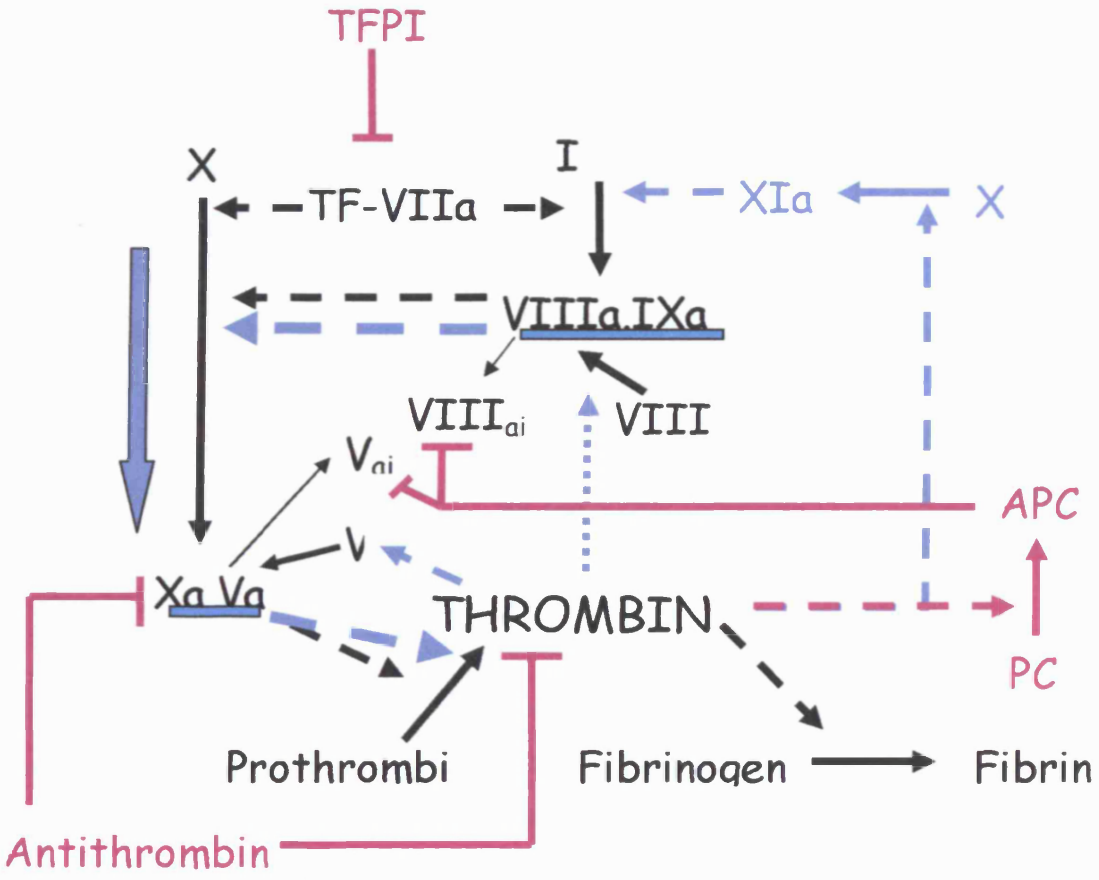


Fig 1.2 Coagulation cascade. Initial thrombin generation in black arrows, explosive thrombin generation in blue, termination of thrombin generation in purple.

1.1.2.4 Thrombin

Pro-thrombin is activated to thrombin in the prothrombinase complex. The first cleavage of pro-thrombin results in the pro-piece and pre-thrombin 2, this is followed by a further cleavge in pre-thrombin 2 to α-thrombin which consists of an A and B-chain which are held together by a disulphide bridge. Further cleavages in the B chain lead to the haemostatically inactive β and γ thrombin forms.

Thrombin contains four binding sites for substrates, inhibitors, cofactors and sodium ions, Sodium ions determines if thrombin is procoagulant (present) or anticoagulant (absent) in which case thrombin activates protein C.

Thrombin is the key serine protease in coagulation by activating fibrinogen to fibrin and hence forming a clot. In addition, thrombin also activates FV, FVII, FVIII and FXI in the clotting cascade causing an explosion of thrombin generation. In addition thrombin activates FXIII and TAFI which lead to clot stabilisation. Thrombin cleaves after arginine ester and amide bonds in both natural and artificial substrates by forming an intermediate covalent tetrahedral complex (Hemker, 1983).

Thrombin is inhibited by a number of proteins, AT and α2-macrogloublin are the main inhibitors accounting for 64 and 23% respectively (Hemker *et al*, 1986;Jesty, 1986). α2-macrogloublin comprises of four identical subunits, which consists of two pairs of subunits which are held together by disulphide bonds, with a plasma concentration of 3μM. α2-macrogloublin is a non-specific inhibitor if proteinases. Proteinases bind to the "bait" region of α2-macrogloublin, this causes a conformational change in the α2-macrogloublin complex which traps the proteinase. However, the active site of proteinase is not involved in binding to α2-macrogloublin, therefore small substrates can still be cleaved (Ganrot, 1967).

Synthetic substrates

Synthetic substrates for the detection of thrombin consist of a tri or tetrapepetide to which a chromophore or fluorophore is attached at the caboxy terminal via an amide bond. When

the enzyme of interests cleaves this bond the chemophore is released, and this has an absorption maximum at a different wavelength to that of the whole substrate. Fluorogenic substrates are ten fold more sensitive than chromogenic (Hemker, 1983).The first chromogenic substrate for thrombin was Bz-Phe-Val-Arg-pNA (Blomback *et al*, 1974). There are now many substrates which include H-D-Phe-Pip-Arg-pNA (S-2238), Tos-Gly-Pro-Arg-pNA (Chromozyme-TH)

1.1.2.5Thrombin generation

Initially thrombin is generated through the extrinsic pathway, this enables low levels of thrombin to be generated. Only a minute amount of thrombin 0.5-2nM is necessary to activate platelets, FXIII, FV and cause fibrin formation (Brummel *et al*, 2002). In a computational model of coagulation the intrinsic complex was at a higher concentration than the extrinsic at 300 seconds (Hockin *et al*, 2002). The bulk of thrombin is generated in the propagation phase which accounts for 96% of the total thrombin generated (Brummel *et al*, 2002), this thrombin is generated mainly by the intrinsic pathway because TF-VIIa is rapidly inactivated by TFPI (Baugh *et al*, 1998).

1.1.2.6 Inhibition of thrombin generation

Protein C and S are key components of the anti-coagulant pathways which bring coagulation to an end. They also prevent coagulation from occurring in the absence of damage to the vessel wall. When thrombin binds to the endothelial cell membrane protein, thrombomodulin, this causes a change in thrombin function from pro-coagulant to anti-coagulant (Esmon *et al*, 1982;Ye *et al*, 1992). The thrombomodulin-thrombin complex is able to activate protein C to activated protein C (APC) which inactivates FVIIIa and FVa. In addition, the light chain of FVa acts as a cofactor in thrombin activation of APC (Salem *et al*, 1983). The action of APC on inactivation of FVIIIa and FVa is augmented by protein S, which acts as a cofactor (Walker, 1980).

Another important component of coagulation inhibition is the action of antithrombin (AT) which is enhanced in the presence of heparin. AT can inactivate FXa (Yin *et al*, 1971), FIXa (Rosenberg *et al*, 1975) and thrombin (Machovich *et al*, 1976). TFPI suppresses

activation of FX and FIX by TF-VIIa by forming a quaternary complex with FXa, FVIIa and TF in the presence of Ca^{2+} (Broze, Jr. *et al*, 1988).

1.1.2.7 Clot stabilisation

The final stage of the coagulation mechanism is the formation of a fibrin clot. Thrombin cleaves fibrinogen to form fibrin. Fibrinogen comprises of three chains held in a dimeric conformation $A\alpha B\beta\gamma$, thrombin cleaves the N-terminal of $A\alpha$ which allows binding of the γ chain of another fibrin monomer. Thrombin also cleaves the N-terminal of the $B\beta$chain which allows branching to form. In addition, thrombin activates FXIII, which stabilises the fibrin network by creating cross-links between the γ chains of adjacent fibrin monomers. Each monomer can form up to six cross-links with neighbour monomers, thus an insoluble fibrin network is created

1.1.3 Fibrinolysis

The clot structure is broken down by the process of fibrinolysis. Plasmin is the main fibrinolytic agent *in vivo*, however, it circulates in the inactive form plasminogen which is activated by tPA (tissue type plasminogen activator) or uPA (urinary type plasminogen activator). Plasmin hydrolyses peptide bonds in fibrin to create two degradation products, Fragment D and E, which are soluble. Plasmin is inhibited by α_2-antiplasmin, although while bound to fibrin, plasmin is protected from inactivation. FXIIIa cross-links α_2-plasmin inhibitor to α chains of fibrin, thereby increasing resistance to degradation. In addition, FXI can also inhibit clot lysis with as little as 0.01% FXIa required to totally inhibit fibrinolysis (von dem Borne *et al*, 1995).

1.1.4 Platelets

Platelets are small anucleated cells, which are derived from the cytoplasm of megakarocytes. Platelets circulate within the blood at 150-400 x 10^9/L and have a life span of 7-10 days. Activation of platelets by soluble agonists such as adenosine diphosphate (ADP) and thrombin, or by adhesion leads to shape change with formation of pseudopods that represent protrusions of the plasma membrane, in this way the surface area of the platelet increases.

1.1.4.1 Platelet structure

Platelets consist of a cytoplasmic membrane, a structural zone, organelles and a membrane system. The structural zone consists of microtubuli, and the structural proteins, actin and actin binding protein. The function of these are to maintain the resting discoid shape, and actively participate in the shape changes of activated platelets. The organelles consist of mitochondria, glycogen stores and three storage granules: α granules, dense bodies and lysosomes. The membrane system consists of the open canalicular system and the dense tubular system. The dense tubular system is one of the main storage sites for intracellular calcium and is derived from the rough endoplasmic reticulum of the megakaryocyte.

The cytoplasmic membrane is a phospholipid bilayer containing phosphatidylcholine 38%, phosphatidylethanolamine (PE) 27%, sphingomyelin (SPH) 17%, phosphatidylserine (PS) 10% and phosphatidylinositol (PI) 5%. However the phospholipid composition of the bilayer is asymmetrical, the negatively charged phospholipids are almost exclusively on the inner membrane (PE, PS, PI, see Table 1.1), which is maintained by an active process. The organisation of the plasma membrane "flip-flops" during activation to expose the pro-coagulant negatively charged phospholipids. The exposure of procoagulant phospholipids is mediated through the intracellular rise of Ca^{2+} and subsequent signalling pathways (Martinez *et al*, 1999;Heemskerk *et al*, 1997).

Table 1.1 Phospholipid composition (percentage) of the exterior and interior platelet membranes. Adapted from Williams Haematology (Ware & Coller, 1995).

	Exterior Leaflet	Interior Leaflet
PC	45	55
SPH	93	7
PE	20	80
PI	16	84
PS	9	91

1.1.4.2 Adhesion

In intact blood vessels, endothelial cells line the procoagulant subendothelial matrix and in the resting state exert an anticoagulant effect through the action of thrombomodulin. At a site of vascular damage endothelial cells are removed and procoagulant collagen and fibrinonectin are exposed to flowing blood. Endothelial cells may also become procoagulant through inflammatory pathways to expose P-selectin. Circulating VWF attaches to collagen to create a surface on which platelets can attach. Platelets are also able to bind through the glycoprotein (GP) VI receptor to exposed collagen. Platelets attach through the receptor complex GPIb-IX-V to P-selectin on activated endothelial cells or to VWF. Platelets can then roll along this surface by making new contacts through the GPIb-IX-V complex in the direction of flow and dissociating previous interactions. Platelets can detach at this stage and return to normal blood flow or attach through binding of the GPIIb-IIIa.

The manner in which platelets adhere depends upon the levels of shear stress in the vessel, with more platelet tethering occurring with increasing shear stress. Platelets tether through interaction with $GPIb_{\alpha}$, although only 60% of platelets tether through this mechanism at low shear levels. At low shear rates the interactions of the platelet with collagen, fibrinonectin and fibrinogen are sufficient to withstand the shear stress. In addition, release at low shear of endothelium VWF from the Weible-Palade bodies can recruit platelets to the endothelium surface through interactions with the $GPIb_{\alpha}$ receptor (André *et al*, 2000). Finally the integrin $\alpha_{IIb}\beta_3$ is necessary for stationary adhesion contacts at all shear levels (Kulkarni *et al*, 2000).

1.1.4.3 Activation

Once platelets are bound to the vessel surface they can be activated by a variety of ligands, most notably thrombin. Thrombin can activate platelets through protease associated receptors (PAR). There are four PARs, although only 1 and 4 are found on human platelets (3 and 4 in mice). Thrombin binds to the N-terminal end of the PAR receptor and cleaves

the N-terminal, this then allows the remaining N-terminal to interact with the receptor and activate internal messengers and calcium release through the receptor associated G proteins (Vu *et al*, 1991).

It is thought that the PAR1 receptor alone is necessary for platelet response at low thrombin concentrations, as blocking PAR4 still resulted in full platelet activation (Kahn *et al*, 1999). However, PAR4 does seem necessary for a more intense intracellular signal. Khan et al blocked both PAR1 and PAR4 which abolished platelet aggregation through thrombin activation (Kahn *et al*, 1999). There is also a thrombin binding site on $GPIb_{\alpha}$ although the exact role of this interaction in platelet activation is uncertain. Dubois et al following desensitisation of the PAR receptors reported that platelet aggregation still occurred through thrombin binding to the GPIb-IX-V complex and was able to induce platelet aggregation through binding of fibrin to GPIIa-IIIb (Dubois *et al*, 2003). PAR-1 can be activated by FXa in a manner which is independent of thrombin, although high concentrations of FXa are required (Riewald *et al*, 2001), this FXa however, may not activate platelets, but rather produce a proinflammatory response (Riewald & Ruf, 2001).

Adhesion and activation of the platelet through collagen requires both the GPVI receptor and integrin $\alpha_2\beta_1$. When platelets are activated, shape change occurs, the platelet flattens, and the membrane flip-flops exposing procoagulant phospholipids on the platelet surface. This exposure is necessary for the generation of more thrombin and provides a surface on which more platelets are able to aggregate leading to the formation of a thrombus.

1.1.4.4 Thrombus propagation

Platelets translocate on the subendothelial VWF then arrest and recruit additional platelets into growing thrombi. Platelets adherent to collagen expose more PS than platelets adherent to fibrinogen which are less able to support the prothrombinase complex, although this is not a limiting factor if sufficient plasma FVa is available (Briedé *et al*, 2001).

A real time experiment, under shear conditions, found that there was a rapid deposition of platelets and fibrin onto collagen coated glass plates, and that circulating TF was recruited

to areas in which there was a large deposition of platelets and thrombi (Balasubramanian *et al*, 2002). In an *in vivo* model in which injury was induced in the endothelium with a laser, TF was concentrated at the thrombus-vessel wall interface, which suggests that the vessel wall is the source of TF, and not circulating TF (Falati *et al*, 2002).

1.1.5 FVIII

The gene for FVIII is located on the X chromosome. It is a large gene and comprises 1% of the chromosome. The FVIII gene was sequenced in 1984 (Vehar *et al*, 1984;Wood *et al*, 1984;Gitschier *et al*, 1984). FVIII comprises two A domains one B domain, two C domains and three acidic regions (Fig 1.3).

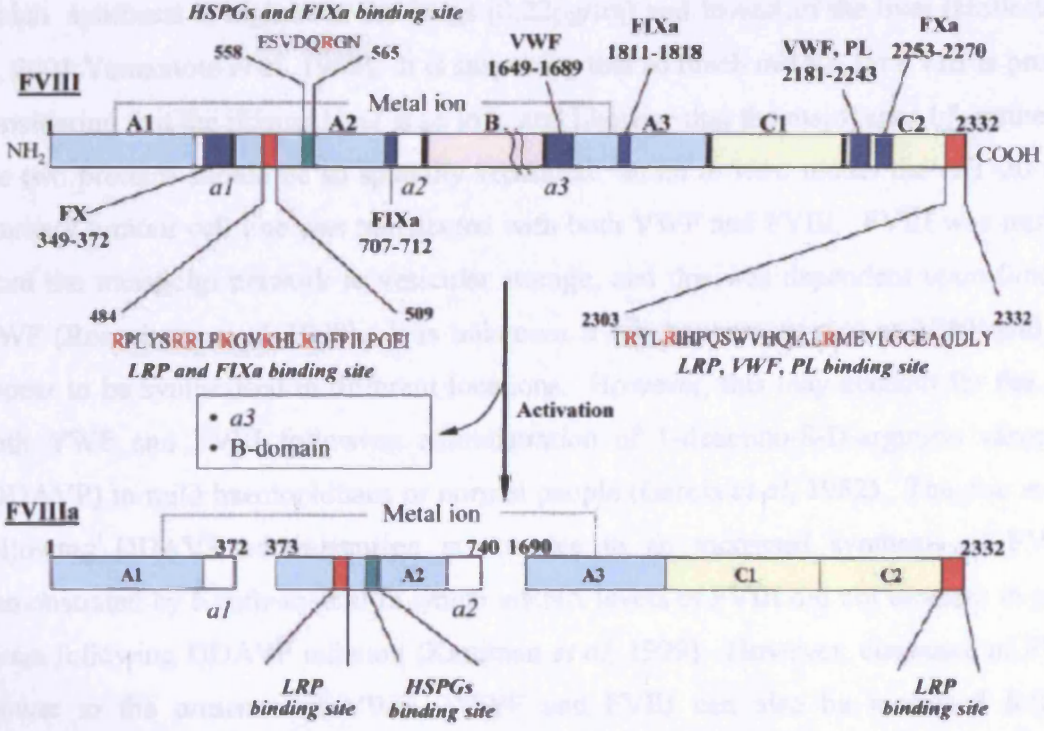


Fig 1.3 Structure of FVIII showing binding sites and activation cleavage sites. Taken from (Saenko et al, 2000).

1.1.5.1 Synthesis

FVIII is synthesised in a wide range of tissues, which includes the liver, kidney, spleen, and muscle (Wion *et al*, 1985;Hollestelle *et al*, 2001). Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) in mice has shown similar mRNA levels of 257 and 209 pg/μg total RNA for liver and kidney respectively, which would account for 75 and 12.5% of plasma FVIII respectively (Hollestelle *et al*, 2001). Liver transplantation in haemophiliacs also corrects FVIII level (Bontempo *et al*, 1987), which supports the idea that the liver is the primary site of FVIII synthesis. A number of studies have found that FVIII is produced in sinusoidal endothelial cells and not in hepatocytes (Stel *et al*, 1983;Do *et al*, 1999;Zelechowska *et al*, 1985;Hollestelle *et al*, 2001;Hollestelle *et al*, 2004).

The concentration of FVIII mRNA is much higher than that of the carrier protein VWF, for which synthesis is highest in the lungs (0.22pg/μg) and lowest in the liver (Hollestelle *et al*, 2001;Yamamoto *et al*, 1998). It is surprising that so much mRNA for FVIII is produced considering that the plasma level is so low, and likewise that the major sites of synthesis for the two proteins should be so spatially separated. In an *in vitro* model the AtT-20 mouse pituitary tumour cell line was transfected with both VWF and FVIII. FVIII was trafficked from the transgolgi network to vesicular storage, and this was dependent upon functional VWF (Rosenberg *et al*, 1998). It is unknown if this happens *in vivo* as VWF and FVIII appear to be synthesised in different locations. However, this may account for the rise in both VWF and FVIII following administration of 1-deamino-8-D-arginine vasopressin (DDAVP) to mild haemophiliacs or normal people (Garcia *et al*, 1982). The rise in FVIII following DDAVP administration is not due to an increased synthesis of FVIII as demonstrated by Kaufman et al in which mRNA levels of FVIII did not increase in porcine livers following DDAVP infusion (Kaufman *et al*, 1999). However, clearance of FVIII is slower in the presence of VWF. VWF and FVIII can also be increased following administration of interluekin-11, however, this occurs steadily over time, and in a manner which is different to DDAVP increases (Olsen *et al*, 2003).

1.1.5.2 Secretion of FVIII

The initial stage of secretion involves translocation of the mature full length amino acid polypeptide into the lumen of the endoplasmic reticulum (ER), where glycosylation occurs. Transfer of FVIII from the ER to the Golgi is not yet fully elucidated, but is mediated in part by the ER-Golgi intermediate compartment (ERGIC). Mutations in ERGIC-53 result in a rare homozygous condition in which both FV and FVIII levels are reduced to between 5-30% of normal (Nichols *et al*, 1999;Neerman-Arbez *et al*, 1999). ERGIC-53 recruits correctly folded FVIII through interactions with N-linked glycosylation in the B domain into the secretory vesicles of the ERGIC for trafficking to the golgi (Moussalli *et al*, 1999). However, mutations in ERGIC-53 do not account for all cases of the combined deficiency and recently another chaperone protein known as multiple coagulation factor deficiency 2 has been implicated (Zhang *et al*, 2003). There is also possibly a third protein involved in this pathway. FVIII also undergoes further processing and intracellular proteolysis (Kaufman *et al*, 1988), in which the B domain is cleaved at the motif Arg-X-X-Arg, resulting in two cleavages at Arg 1313 and 1648. These cleavages give rise to the heterodimeric form of FVIII which circulates in plasma.

The dimeric form of FVIII is held in a stable conformation by a metal ion bridge, as the metal ion chelating agent ethylenediaminetetraacetic acid (EDTA) causes subunit dissociation (Eaton *et al*, 1987). This has more recently being identified as a copper ion with a 1 mol:mol ratio (Tagliavacca *et al*, 1997). The copper binding site has been proposed at Cys 310, with His 99 also being important for the A1-A3 interaction (Tagliavacca *et al*, 1997). Copper increases the intersubunit affinity by a 100-fold, although calcium is necessary for FVIII activity (Wakabayashi *et al*, 2001). Copper has an inhibitory effect on the heavy chain, whereas on the light chain it increases the rate of reconstitution of the subunits and enhancement in the reconstituted FVIII activity (Sudhakar & Fay, 1998).

1.1.5.3 Activation of FVIII

FVIII circulates in plasma in the heterodimeric form bound to the carrier protein VWF which protects against FVIII degradation. FVIII binds to VWF at two sites in the light

chain, the acidic region a3 and in the C2 domain (Saenko & Scandella, 1997). While FVIII is unactivated and bound to VWF it is unable to partake in the tenase complex. *In vitro* FVIII can be activated by both factor Xa and thrombin by cleavage at three arginine residues 372, 740 and 1689. However it would appear that thrombin is the main activator *in vivo* as thrombin activated FVIIIa has a higher activity than that formed by FXa activation (Neuenschwander & Jetsy, 1988;Parker *et al*, 1997).

1.1.5.3.1 Activation FVIII by FXa

FXa can activate FVIII causing a 1.6 fold increase in coagulant activity, however this activation is inhibited by VWF (Koedam *et al*, 1990). This is because the binding site for FXa overlaps that of the VWF binding site (Nogami *et al*, 1999). Work has since shown that the C2 domain contains a FXa binding site between residues 2253-2270, whereas the FXa cleavage site is in the A3 domain (Hay, 1999). In addition the PL binding site is also contained within the C2 domain (Scandella *et al*, 1995). These findings, suggest that FVIII activation by FXa is unlikely to occur under normal haemostatic conditions.

1.1.5.3.2 Activation of FVIII by thrombin

Activation of FVIII by thrombin occurs by three further cleavages of the FVIII dimer at Argenine 372, 740 and 1689. Pitman et al demonstrated by amino acid subsitution that Arg 740 did not appear to be necessary for FVIII activity, whereas Arg 372 was the main site for activity, and Arg 1689 was also necessary (Pittman & Kaufman, 1988). Using snake venoms to cleave the heavy chain site, Arg 1689 was not required in the absence of VWF however, when VWF was present, cleavage in both heavy and light chain was necessary (Hill-Eubanks *et al*, 1989). This would suggest that cleavages at Arg 372 and 1689 are required for full FVIII activity (Regan & Fay, 1995). Cleavage at Arg 1689 appears to be the mechanism by which VWF disassociates from the FVIII molecule. VWF promotes this cleavage by increasing the catalytic efficiency of thrombin cleavage by a factor of seven (Hill-Eubanks & Lollar, 1990). Therefore, Arg 372 appears to be the limiting step in FVIII activation. Cleavage at Arg 1689 cleaves the acidic region a3 from the light chain of FVIII which affects the conformation of the C2 domain. This altered conformation in the C2 domain then allows for VWF dissociation (Nogami *et al*, 2000), thereby increasing the

affinity of FVIII for PL by eight fold (Saenko *et al*, 1998). The thrombin binding site for this cleavage is contained within the C2 domain, but is separate from VWF (Nogami *et al*, 2000) and PL binding sites (Pratt *et al*, 1999). There is however, overlap in the PL and VWF binding sites (Saenko *et al*, 1994) and FXa binding site (Nogami *et al*, 1999).

The fully activated FVIII is in a trimeric form with the A1 and A3-C1-C2 domains held together by metal ions (Tagliavacca *et al*, 1997), the A2 domain is loosely associated with the complex by weak electrostatic charges (Lollar & Parker, 1990;Fay *et al*, 1991b).

1.1.5.4 Tenase complex

FVIIIa acts as a cofactor in the tenase complex in which FX is activated to FXa by FIXa on a PL surface (Fig 1.4). FVIIIa increases the catalytic efficiency of this complex by several orders of magnitude (Kane & Davie, 1988). FVIIIa binds to FIXa in a 1:1 complex (Lamphear & Fay, 1992) through the A2 and A3 domains (Mertens *et al*, 1999) to the EGF (Christophe *et al*, 1998) and protease domain (Bajaj *et al*, 1985) of FIXa. The FVIIIa-FIXa complex has high affinity with a 0.5nM dissociation constant (Jesty, 1990), which is also dependent upon calcium (Duffy *et al*, 1992). It appears that the A2 domain of FVIIIa modulates FIXa activity (Jenkins *et al*, 2002). However, the other components of the complex also have important roles. FX increases the affinity of FIXa for FVIIIa (Mathur *et al*, 1997). Also it would seem that FVIIIa and FX increase the affinity of receptors on the surface of activated platelets for FIXa by five fold (Ahmad *et al*, 1989). The PL surface also has two functions PS and PE are necessary for FVIIIa binding to the PL surface (Gilbert & Arena, 1995), and the PL surface also contributes to the complex by increasing the catalytic efficiency by 1000 fold (Gilbert & Arena, 1996). The binding of FIXa and FVIIIa to PL membranes is a mechanism by which the local concentrations of the factors will be higher allowing for interaction between the two proteins.

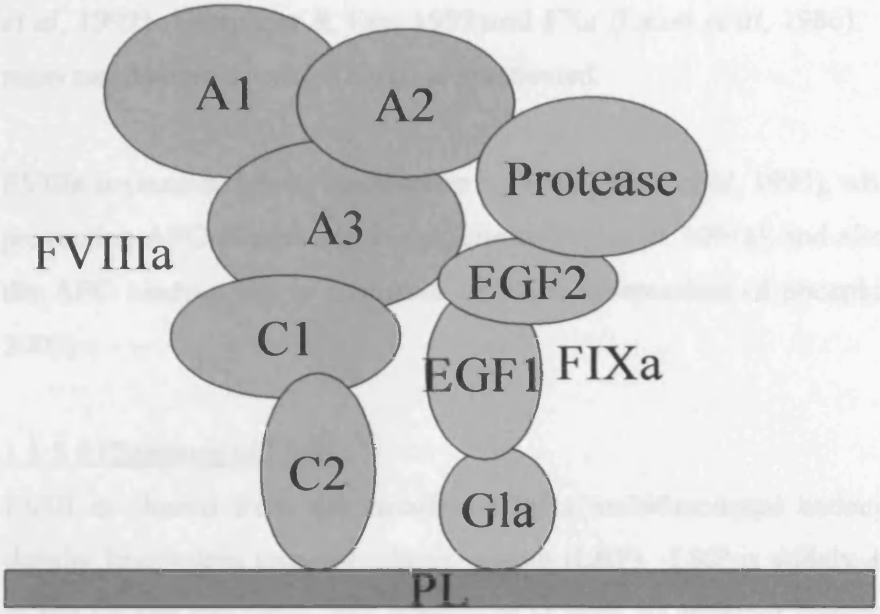


Fig 1.4 Interaction of FVIIIa and FIXa. FVIIIa and FIXa bind to a PL surface through the C2 and Gla domains respectively. FVIIIa binds to FIXa through interactions with the A2 and A3 domain of FVIIIa and the EGF2 and Protease domain of FIXa in a 1:1 ratio.

Metal ions are also key components of the tenase complex. Magnesium ions facilitate efficient FXa generation with much lower amounts of FVIIIa than those with calcium alone. Ions also potentiate the activation of FIX by FXIa, and also activation of FX by FIXa (Sekiya *et al*, 1996).

1.1.5.5 Inactivation of FVIII

FVIII can be inactivated by a number of different processes. The A2 subunit of the heterotrimeric form is weakly associated with the dimer, disassociation of the A2 subunit results in loss of FVIIIa activity (Lollar & Parker, 1991;Fay *et al*, 1991b).

FVIII is also inactivated by two further cleavages of the heterotrimer at Arg 336 and 562 by APC (Fay *et al*, 1991c). Protein S acts as a co-factor in this reaction and increases the inactivation rate nine fold (Koedam *et al*, 1988), Factor V has also found to be a cofactor in this reaction (Shen *et al*, 1997). In addition Arg 336 can also be cleaved by FIXa (O'Brien

et al, 1992) (Lamphear & Fay, 1992)and FXa (Eaton *et al*, 1986). However, APC is the main mechanism by which FVIII is inactivated.

FVIIIa is protected from inactivation by VWF (Rick *et al*, 1990), which protects FVIIIa by preventing APC-phospholipid interactions (Fay *et al*, 1991a), and also by directly inhibiting the APC binding site in a manner which is independent of phospholipids (Nogami *et al*, 2002).

1.1.5.6 Clearance of FVIII

FVIII is cleared from the circulation by a multifunctional endocytic receptor, the low density lipoprotein receptor-related protein (LRP). LRP is widely expressed on a number of tissues and cell types, and recognises at least 30 different ligands, several of which are involved in haemostasis (Herz & Strickland, 2001). FVIII interacts with LRP through the C2 (Lenting *et al*, 1999) and A3 domains of the light chain (Bovenschen *et al*, 2003), and also the A2 domain of the heavy chain (Saenko *et al*, 1999b). Clearance of FVIII by LRP can be prevented by receptor associated protein (RAP) (Lenting *et al*, 1999;Saenko *et al*, 1999b), which is an antagonist for all ligands to LRP. Heparan sulfate proteoglycans (HSPGs) also facilitate in clearance mediated through LRP, and also in a non-LRP mediated clearance (Sarafanov *et al*, 2001).

1.1.6 Von Willebrand Factor

1.1.6.1 Synthesis, secretion and storage

VWF is a 52 exon gene (Mancuso *et al*, 1989) which is located on chromosome 12 (Ginsburg *et al*, 1985). Synthesis of VWF occurs in two different cell types, in endothelial (Jaffe *et al*, 1973;Wagner *et al*, 1982) and in megakaryocytes (Nachman *et al*, 1977), which are the precursor cells to platelets. VWF is stored within these cells in the Weibel-Palade bodies of endothelial cells (Wagner *et al*, 1982;Wagner, 1993), and the α-granules of platelets (Cramer *et al*, 1985). VWF is secreted constitutively from endothelial cells giving rise to the plasma source of VWF. Stored VWF is released as very high multimers (Sporn *et al*, 1986) which are haemostatically more effective than low molecular weight multimers.

VWF is synthesised as a large prepro-peptide, which is in total 2813 amino acids long, this consists of a signal peptide and 4 repeated domains A-D. The signal peptide is cleaved during translocation into the ER, the resulting pro-VWF forms dimers through disulphide bridges at the carboxy termini (Voorberg *et al*, 1991). Within the trans Golgi network sulphation occurs followed by multimerisation through the amino terminal and cleavage of the propeptide (Carew *et al*, 1990;Vischer & Wagner, 1994) which increases FVIII binding to VWF (Bendetowicz *et al*, 1998). The propeptide is required for multimerisation of VWF and transport to the Weibel-Palade bodies (Journet *et al*, 1993), which were shown to be independent of each other (Haberichter *et al*, 2000).

1.1.6.2 FVIII-VWF complex

Theoretically VWF and FVIII can bind in a 1:1 ratio as there is 1 FVIII binding site per VWF monomer (Marti *et al*, 1987), however, in plasma a 50:1 ratio has been observed (Vlot *et al*, 1995). VWF stabilises FVIII in plasma and prolongs the half-life (Weiss *et al*, 1977;Over *et al*, 1978), this is achieved by two mechanisms, firstly by preventing FVIII binding to PL before activation by thrombin (Saenko & Scandella, 1995;Hill-Eubanks *et al*, 1989), and secondly by preventing inactivation by APC (Nogami *et al*, 2002). The FVIII-VWF complex forms rapidly, with more than 95% of infused FVIII bound to VWF in 12 seconds (Vlot *et al*, 1996).

1.1.6.3 VWF/Platelet interactions

VWF interacts with two receptors on the surface of platelets, glycoprotein GPIbα in the GPIb-IX-V complex and intergrin $\alpha_{IIb}\beta_3$. The GPIbα is the most important interaction at high shear, at low shear this interaction is not necessary, and platelets are tethered though interaction with collagen, fibronectin, and fibrinogen/fibrin (Savage *et al*, 1998). VWF binding to GPIbα slows platelets down and allows sufficient time for platelets to make stable interactions through other receptors (Savage *et al*, 1996). At high shear GPIbα interaction with VWF may also be important for initiating platelet activation.

1.2 Haemophilia

Haemophilia is a bleeding disorder resulting from a congenital deficiency in FVIII (Haemophilia A) or FIX (Haemophilia B). The first written evidence of haemophilia is in the Talmud in the 5th century AD and in subsequent writings of the Rabbis. It was advised that circumcision should not occur if two siblings had died from bleeding, it was also noted that this rule was to be followed even if the woman had more than one husband, which acknowledges the female transmission of the bleeding disorders (Rosner, 1969). Haemophilia A is a genetic disorder in which males are affected because the FVIII gene is carried on the X chromosome. In some cases carriers may also be affected by low levels of FVIII. From hereon in deficiency in FVIII will be termed haemophilia.

1.2.1 Genetic defect

Haemophilia is caused by a mutation in the FVIII gene. Mutations causing a point mutation can have several outcomes, a missense mutation in which a different amino acid is coded, a nonsense mutation in which a stop codon is produced leading to termination of the protein, or a mutation may affect the splicing sites between the exons. The largest group of point mutations are missense which account for 81% of this group (Kemball-Cook & Tuddenham, 2003). Patients with missense mutations can be cross reactive material (CRM) reduced or negative if the amount of FVIII antigen is reduced in proportion with the FVIII activity, or CRM +ve if the amount of FVIII antigen is normal but the FVIII activity is reduced (Kemball-Cook & Tuddenham, 2003). Patients who are CRM+ve have a normal expression of the FVIII protein, but presumably the mutation has affected interactions with the other coagulation proteins. Mutations can also result in a insertion or deletion, an increase or decrease in three bases maintains the reading frame, where as other deletions will alter the reading frame and a stop codon will be produced causing a premature termination of the FVIII protein. The most common mutation in severe patients is the intron 22 inversion which accounts for around 50% of cases (Antomarakis, 1995).

1.2.2 Symptoms and FVIII level

The normal FVIII range is considered to be 50-150%. Haemophilia patients have FVIII levels less than 50%, these are divided into three categories depending upon their FVIII level, which in general correlate with their clinical symptoms.

1.2.2.1 Elevated FVIII levels

There is some evidence that high levels of FVIII greater than 150% can act as an independent risk factor for thrombosis, odds ratio of 4.8 after adjusting for blood group and VWF (Koster *et al*, 1995). In an animal model Kawasaki et al demonstrated that elevated plasma FVIII levels led to an increase in FVIII concentration within a thrombus, and that VWF participated in this accumulation (Kawasaki *et al*, 1999). The increase in FVIII levels are due to an increase in protein secretion or in stability (O'Donnell *et al*, 1997), so far no genetic cause has been identified with screening of the promoter and the poly A site (Mansvelt *et al*, 1998). There is a high heritability for FVIII and VWF, which is due in part to blood group, those with blood group O have lower FVIII and VWF concentrations (de Lange *et al*, 2001).

1.2.2.2 Reduced FVIII levels

Mild haemophilias are classified according to the International Society of Thrombosis and Haemostasis (ISTH) as having 5-40% of normal, moderate as 1-5% and severe as having less than 1% of normal, 100% of normal is equivlent to 1 IU/ml (Table 1.2). Mild/moderate haemophilia can be caused by a variety of genetic causes; impaired FVIII synthesis, processing or secretion, reduction of FVIII binding to VWF, impaired thrombin activation, impaired FVIII stability due to accelerated dissociation of the A2 domain, missense mutations at PL binding surfaces, and altered interaction with FIXa (Jacquemin *et al*, 2003).

Table 1.2 Classification of haemophilia, taken from (Bolton-Maggs & Pasi, 2003).

Concentration of FVIII	Classification	Clinical
<0.01 IU/ml	Severe	Spontaneous joint and muscle bleeding; bleeding after injuries, accidents and surgery
0.01-0.05 IU/ml	Moderate	Bleeding into joints and muscles after minor injuries; excessive bleeding after surgery and dental extractions
>0.05-0.4 IU/ml	Mild	Spontaneous bleeding does not occur; bleeding after surgery, dental extractions, and accidents

Those with haemophilias have a bleeding time which is the same as normal individuals, the bleeding tendency is therefore not due to a prolongation in the time taken to clot, rather bleeding occurs after a delay. In a morphological study of haemophilic clots it was observed that the fibrin plug was thinner than normals and contained red blood cells and leukocytes within the centre of the plug (Sixma & van den Berg, 1984). This suggests, the defect in haemophilia is due to an inability to form stable fibrin clots.

The role of TAFI may be a key in the inability to form stable fibrin clots. Monsnier et al demonstrated that at low TF concentrations, not enough thrombin is produced to activate TAFI, whereas at high TF concentrations enough thrombin was generated by the extrinsic pathway to activate TAFI independent of the intrinsic system (Mosnier *et al*, 2001). This suggests that in areas with low TF concentrations the clot may be more susceptible to fibrinolysis. The amount of TF is low or absent in the skeletal muscle and in connective

tissues at joints (Drake *et al*, 1989), which corresponds to those areas in which spontaneous bleeding occurs in severe haemophilia. Additionally Carr et al have demonstrated that in FVIII deficient blood a decreased platelet contractile force is generated which is half that of normal plasma. A consequence of this reduced force is a reduction in the elasticity of the clot, which is another mechanisms by which haemophilic clots are more prone to fibrinolysis (Carr, Jr. *et al*, 2002).

1.2.3 Inhibitors

Inhibitors are the main complication of haemophilila as an inhibitor is an antibody which neutralises FVIII activity by preventing interactions with VWF (Saenko & Scandella, 1997), PL (Barrowcliffe *et al*, 1983), FIXa (Fijnvandraat *et al*, 1998;Zhong *et al*, 1998), thrombin (Precup *et al*, 1991), and FXa (Lollar *et al*, 1994;Foster *et al*, 1988). Inhibitors can also cause proteolysis of FVIII (Lacroix-Desmazes *et al*, 1999;Lacroix-Desmazes *et al*, 2002). Epitope mapping studies have shown that inhibitors tend to have restrictive binding sites, predominantly on the A2, C2 and A3 domains on the FVIII molecule (Arai *et al*, 1989;Lenting *et al*, 1996;Zhong *et al*, 1998;Scandella *et al*, 1995;Scandella *et al*, 1995;Prescott *et al*, 1997). The highest inhibitor titres are found towards the light chain of FVIII (Scandella *et al*, 2001). Moreover, recent studies have shown that some inhibitors are directed also towards the acidic –a1, -a2 and –a3 regions (Barrow *et al*, 2000;van den Brink *et al*, 2000;Raut *et al*, 2003b). It is well known that these acidic-regions are specific for proteolytic cleavages via thrombin or FXa and thus constitute important functional sites, particularly in the activation and inactivation of FVIII. Furthermore, various models (Pemberton *et al*, 1997;Kemball-Cook *et al*, 1998;Stoilova-McPhie *et al*, 2002) have predicted that these regions are exposed on the surface of the FVIII molecule and therefore may be open to a number of surface proteins.

Inhibitors to FVIII are measured using the Bethesda assay in which the sample plasma is incubated with an equal volume of pooled normal plasma for two hours at 37°C. The sample and normal plasma mixture as well as a control are assayed for FVIII as normal. If an inhibitor is present then residual FVIII will be less than the control, a reduction of 50% in a two hour incubation period is the definition of one Bethesda unit (Kasper *et al*, 1975).

In haemophilic patients the rate of inhibitor development has been linked to the type of genetic mutation and a higher incidence is found in those with null mutations. Inhibitors affect 24-33% of severe-moderate patients (Bray *et al*, 1994;Addiego *et al*, 1993;Ehrenforth *et al*, 1992). Inhibitors can also arise in mild patients, although the risk is a quarter of those with severe haemophilia (Hay, 1998). There is some evidence to suggest that in mild haemophilias inhibitors can develop following intense FVIII exposure (Sharathkumar *et al*, 2003).

Two inhibitor outbreaks have been documented following a change in the viral inactivation process used (Peerlinck *et al*, 1993;Rosendaal *et al*, 1993;Peerlinck *et al*, 1997). This product showed some unique characteristics, increased thrombin proteolysis, more rapid FXa generation and enhanced phospholipid binding (Barrowcliffe *et al*, 1993a;Raut *et al*, 1998).

Inhibitors can also arise in patients without a genetic defect, which is termed acquired haemophilia. Acquired haemophilia is idiopathic in around 60% of cases, but can be associated with malignancy, post partum and autoimmune disorders (Delgado *et al*, 2003). FVIII antibodies have also been detected in 17% of normal healthy people (Algiman *et al*, 1992;Moreau *et al*, 2000). There are also studies which show that FVIII antibodies in both normal and haemophilia patients can be neutralised by anti-idiotypic antibodies(Sultan *et al*, 1984;Gilles & Saint-Remy, 1994;Gilles *et al*, 1996).

1.2.4 Treatment

1.2.4.1 FVIII replacement and prophylaxis

Treatment of haemophilia is by replacing the missing or dysfunctional FVIII. In patients with mild or moderate haemophilia this would be after injury or before a surgical procedure. Severe haemophilias can bleed spontaneously into the joints so require treatment more often. Standard treatment used is on demand therapy using a FVIII concentrate as required; however, prophylaxis is now more widespread, especially in

children and has several advantages. By maintaining FVIII at a low level spontaneous bleeding into the joints can be avoided. Which when started at a young age prevents the development of arthropathy (van den Berg *et al*, 2001;Astermark *et al*, 1999). More details on FVIII concentrates are given in section 1.2.4.3

1.2.4.2 FVIII bypassing agents

A major challenge to the treatment of haemophilia is the development of inhibitors to FVIII. Treatment of inhibitor patients can be by immune tolerance in which large amounts of FVIII are given to overcome the inhibitor. Alternatively a product which bypasses FVIII can be given such as the prothrombin converting complex, FEIBA or Autoplex (Lusher, 1994). Despite the usage of these bypassing agents for more than 20 years the mode of action is unclear, although recent work using a purified system found that a combination of 1nM FXa and 35μM prothrombin had the same effect *in vitro* and *in vivo* as FEIBA (Turecek *et al*, 1999). This has been confirmed with a recombinant product showing that the previous finding was not due to contaminants (Himmelspach *et al*, 2002).

A more recent approach to FVIII inhibitors is the administration of high concentrations of recombinant FVIIa (rFVIIa) at concentrations much higher than in normal haemostasis (Seremetis, 1994). Although the mechanism for FVIIa action is not understood there are suggested explanations. Butenas et al demonstrated that in acquired haemophilia B 10nM rFVIIa with an elevated number of platelets and 5pM TF had the same effect on haemostasis as that of normal plasma (Butenas *et al*, 2003). This maybe because there is an increased amount of FXa generation at the platelets surface with elevated rFVIIa levels (Monroe *et al*, 1998). rFVIIa also appears to decrease the permeability of the fibrin clot when FVIII or FIX is absent, this may make the clots of haemophilic plasmas resistant to premature fibrinolysis (He *et al*, 2003).

Porcine FVIII is another possibility, although the patient may develop an inhibitor to this as well. However, a complication of porcine FVIII is the theoretical transmission of zoonoses, and for this reason the manufacture is now discontinued. A recombinant porcine FVIII is now entering clinical trials. A conventional FVIII concentrate high in VWF may be

beneficial as it may protect FVIII from the antibodies in particular those against the light chain (Gensana *et al*, 2001).

1.2.4.3 Factor VIII concentrates

There are essentially two types of FVIII concentrates, plasma derived (PD) and recombinant.

1.2.4.3.1 Plasma derived

Cryoprecipitate

Before the advent of cryoprecipitate, the only treatments available to patient were blood transfusions and fresh frozen plasma. This was limiting due to the large volumes of plasma that were needed to cover a bleeding event. Cryoprecipitate was manufactured by removing the plasma and rapidly freezing it, the plasma would then be slowly defrosted in a fridge, the precipitate was then isolated by centrifugation. This process resulted in FVIII concentration increasing by 16-fold (Pool & Shannon, 1965).

Intermediate purity

Intermediate purity (IP) products are manufactured from cryoprecipitate, which are then further purified by heparin precipitation to remove fibrinogen and fibrinonectin. The FVIII is then precipitated with glycine and sodium chloride and lyophilised, the product is then dry heated as the virucidal step (United Kingdom Haemophilia Centre Doctor's Organisation, 2003). These products contain VWF.

High purity

Monoclonal-antibody purified

High purity (HP) concentrates are also made from cryoprecipitate. Fibrinogen and fibrinonectin are removed by centrifugation, and the remaining FVIII is then subjected to a solvent/detergent treatment to remove viruses. The product is then applied to immunoaffinity column and the solvent and detergent are removed by washing before FVIII is eluted. The eluate is then applied to an ion-exchange column. The resulting FVIII

product contains a small amount of VWF. *Replenate* is manufactured by the same methodology (United Kingdom Haemophilia Centre Doctor's Organisation, 2003). Monoclate is purified by a similar method using an antibody against VWF.

Ion-exchange

The initial steps for this method are the same as for monoclonal-antibody purified. After virus inactivation with solvent and detergent, the product is applied to an anion-exchange resin. The solvent and detergent are removed by washing steps, FVIII is then eluted (Schwinn *et al*, 1989).

1.2.4.3.2 Recombinant

Recombinant products are manufactured from cell lines which have had the FVIII gene inserted; FVIII is then harvested from the cell culture media. These products therefore avoid reliance on plasma and in theory has an increased safety profile.

1st Generation

The first recombinant FVIII products to be manufactured were full-length products which were manufactured with proteins derived from animal and humans in both cell culture and in stabilisation of the final product.

Kogenate

The FVIII gene is inserted into a baby hamster kidney cell line. The resulting FVIII is subjected to a number of purification steps to remove the cell culture media and cells. These steps include, anion exchange, immunoaffinity chromatography and gel filtration. The original formulation of *Kogenate* contained albumin, however it is now manufactured without additional albumin and includes a solvent/detergent viral inactivation step (Schwartz *et al*, 1990).

Recombinate

The rFVIII is expressed in Chinese hamster ovary (CHO) cell line. In addition, a recombinant VWF is inserted into the cell line to stabilise FVIII. FVIII is purified from the

cell media by filtration, immunoaffinity chromatography and a two-stage ion-exchange. The final product does not contain VWF (White *et al*, 1989). The same FVIII is now also manufactured without albumin and is called *Advate*.

2nd Generation

A refinement of the manufacture process was to eliminate the need for human serum albumin (HAS) in the final product, thereby reducing the possibility of transmission of blood borne viruses.

B-domain deleted FVIII

An improvement to the full-length recombinant products was the manufacture of a recombinant product which was the B-domain deleted (BDD), so called second generation recombinant FVIII. The B-domain is not thought to play a role in haemostasis. BDD allowed for a greater expression of FVIII in the cell culture system (Toole *et al*, 1986).

The BDD product is known commercially as *Refacto*- It is produced in a CHO cell line in which the modified 170 kDa FVIII is inserted as a single chain with a link between serine 743 and glutamine 1638 (Berntorp, 1997). The FVIII is harvested from the cell culture medium and subjected to 5 chromotography steps and a solvent-detergent viral inactivation step (Eriksson *et al*, 2001). Another advantageous property of the BDD deleted product is that it does not require stabilisation in the final product by HSA (Osterberg *et al*, 2001) (which is necessary for the full-length products), although HSA is present in the cell culture medium.

BDD FVIII has been compared to full-length FVIII in several studies and found to be comparable (Pitman *et al*, 1993;Sandberg *et al*, 2001). The first clinical study found that the recovery and half-life of BDD was the same as a monoclonal purified PD product, however, the volume of distribution and clearance were statistically higher with BDD (Fijnvandraat *et al*, 1997).

Kogenate-SF

A further development of *Kogenate* was to manufacture this product without HSA in the final product. However in this product animal proteins are present during the cell culture steps.

3rd Generation -Protein free recombinant FVIII

Advate

Manufacture of FVIII without the use of any human plasma products is the latest development for FVIII concentrates which are called third generation. The removal of all human and animal proteins from the manufacture of recombinant products should in theory remove any possible transmission of viruses and prion diseases such as new variant Creutzfeldt Jackob disease, which is of particular concern due to the use of foetal calf serum during cell culture. Removal of all proteins from the manufacture of the product *Recombinate* led to the development of the latest FVIII product *Advate*. In addition, a protein free version of *Refacto* is also entering clinical trials.

1.2.4.4 Future developments of FVIII replacement

Due to the high cost and frequency of treatment, improvements such as extending the half-life of the FVIII molecule are being investigated. This could be done either by increasing the stability such that the A2 subunit cannot dissociate so readily, or by decreasing clearance of the FVIII molecule through LRP mediated clearance. Only about 20% of the global population of haemophilic patients are treated on a regular basis (Jones & Robillard, 2003), due to the high cost of both plasma and recombinant FVIII products. Recently FVIII has been expressed in the milk of transgenic pigs (Paleyanda *et al*, 1997), this could offer an approach by which FVIII could be manufactured both cheaply and on a larger scale.

1.2.4.5 Gene Therapy

A long-term goal of the haemophilia community has been gene therapy. Haemophilia is a good target for gene therapy as there is a large therapeutic window, and only a small

amount of FVIII, as in prophylaxis would prevent those with severe haemophilia from spontaneous bleeding. Gene therapy is the process by which the FVIII gene is administered in a viral vector which is then able to enter cells and express FVIII long-term. Gene therapy has been successful in a number of animal models using adenovirus (Brown *et al*, 2004) and adeno-associated virus (Scallan *et al*, 2003;Sarkar *et al*, 2004). However, to date gene therapy has only shown a small amount of promise in clinical trials (White, 2001). A study of 13 patients with a retroviral vector found small increases in FVIII in nine patients with greater than 1% FVIII, these levels though were not sustained. An increase in pharmacokinetic parameters was found with exogenous FVIII infusion, which would suggest increased endogenous levels and five patients had reduced bleeding tendency (Powell *et al*, 2003). Another clinical trial for FVIII used a non-viral vector to transduce patient fibroblasts; these were then expanded and given back to the patient. A small increase in FVIII level (0.8-4% of normal) was observed in four of six patients (Roth *et al*, 2001). These trials suggest that a small amount of FVIII is produced, however, it is very difficult to detect due to the sensitivity of FVIII assays.

An alternative approach may be to correct the mRNA using splicesome-mediated RNA repair. Chao et al corrected the haemophilia phenotype in F8 knockout mice by using a pre-trans-splicing molecule which could combine endogenous pre-mRNA FVIII of the mouse to form a repaired FVIII mRNA(Chao *et al*, 2003). The disadvantage of this technique is that treatment would need to be individualised to the mutation, which would be cumbersome in haemophilia as there is a wide range of mutations.

1.2.5 Measurement of FVIII

The level of FVIII must be obtained to diagnose and measure the severity of haemophilia, and to measure the potency of the replacement FVIII concentrate. It is also necessary to measure the response to treatment, pharmacokinetic studies and to measure response to gene therapy. In addition measurement of FVIII is also important in diagnosis of von Willebrand disease (VWD).

1.2.5.1 One-stage APTT assay

The one-stage APTT assay was developed in 1953 by Langdell et al (Langdell *et al*, 1953). The basis of this bio-assay is that of an APTT in which a sample is mixed with a source of phospholipid and a contact activator, after an incubation period, calcium is added to start the reaction and the time taken for a clot to develop is recorded. For a FVIII assay various dilutions of a standard and the sample are made in buffer and added to FVIII deficient plasma and then tested. By plotting on a graph the time taken to clot for a known (standard) amount of FVIII the amount of FVIII in the sample can be established. This assay is the most commonly used in the clinical laboratory due to the relatively low cost and automation of the assay.

1.2.5.2 Two-stage assay

The two-stage assay was developed by Biggs et al in 1955 (Biggs *et al*, 1955). The test method involves incubation of serum (to provide FX and FIXa), factor V, phospholipid, a dilution of the test or standard (which is absorbed first by aluminium hydroxide, to remove prothrombin). Calcium is then added and the mixture incubated to allow FXa to be generated, at the end of the incubation period normal plasma (to provide prothrombin and fibrinogen) is added and the time required for the plasma to clot is recorded. This method, however, has not reached widespread use as it is difficult to standardise, it is time consuming to perform and is not readily automated.

1.2.5.3 Chromogenic assay

The chromogenic assay is in effect a two-stage assay. In the first step FVIII is activated by thrombin, the second step is the conversion of FX to FXa by the complex of FVIIIa, phospholipid, FIXa and calcium. FXa is then measured by colour change of a chromogenic substrate (van Dieijen *et al*, 1987). The chromogenic assay has a greater precision than the one stage assay 2-7% coefficient of variation (CV) (Rosen *et al*, 1985;van Dieijen *et al*, 1987), as compared to one-stage assays which have an inter-assay variability of 7-10% (Raut *et al*, 2001). The chromogenic assay is not widely used in the clinical setting due to the high cost of chromogenic substrates. However, this test is used for the labelling of

FVIII concentrates, particularly in Europe as this method is stipulated in the European Pharmacopoeia, and is also recommended by the ISTH for measurement of concentrates.

1.2.6 Standards and units

FVIII assays are comparative in nature. The clotting times obtained from dilutions of test samples are directly compared to those obtained from a standard with a known amount of FVIII. Therefore, to measure the correct potency of a sample it must be assayed against a reference standard of known potency. FVIII potency is measured in International Units (IU), 1 IU is equivalent to 100% of normal. There are two FVIII International Standards (IS), one for plasma, and one for FVIII concentrates. These standards, in addition to numerous other coagulation factor standards have been established by the World Health Organisation (WHO), and are made and maintained by National Institute for Biological Standards and Control (NIBSC). The IS must be maintained over several years, so it is impractical to use them in routine laboratories. Instead the IS is used to calibrate a working standard (British Standard) which is then used to calibrate locally collected normal pools. Although not ideal, it is possible to use in clinical laboratories an uncalibrated large plasma pool with an arbitrarily assigned value of 100% for each coagulation factor.

1.2.7 Assay discrepancies

Discrepancies in FVIII levels obtained by the different assays and use of different standards have been noted for the last 25 years.

The discrepancies between assays have yet to be fully elucidated, and in fact the problem has become more complicated with the introduction of increasingly pure FVIII products and the differences in measurement by manufacturers (chromogenic) and that used by clinical laboratories (one-stage).

There are two FVIII standards in an attempt to overcome some of the assay discrepancies by assaying like vs like, i.e. patient plasma samples against a plasma standard, and concentrates are measured against a concentrate standard. This makes though a further complication by the range of FVIII concentrates available of differing purities, so that a

true "like for like" standard is impractical. The other issue is with post-infusion plasma, where it may be more accurate to think of as concentrate diluted in the patient's plasma, and therefore a concentrate standard diluted in FVIII deficient plasma may in theory led to a more accurate determination of the recovery values and minimise discrepancies.

1.2.7.1 Assays for FVIII concentrates

When assaying very high purity PD concentrates and recombinant products a large variation between laboratories was observed ranging from 19-100% (Barrowcliffe, 2003), the standard at that time was a concentrate standard derived from an intermediate purity product. This would suggest that an intermediate purity product is unsuitable for assays of high purity and recombinant products, implying that separate standards for each type of concentrate would be required. However, further research identified aspects of the assay in which small modifications would improve the reliability with high purity concentrates (Barrowcliffe, 1993b). A haemophilic or artificial FVIII deficient plasma containing normal levels of VWF was required in one-stage APTT assays (Barrowcliffe *et al*, 1993b). Inclusion of albumin in all buffers at a concentration of 1% improved reproducibility. Also all concentrates should be initially diluted to approximately 1 IU/ml. In addition, the choice of prediluent was shown to affect potency measurement. With concentrates it was found that haemophilic or artificially depleted FVIII deficient plasma was required to obtain the correct potency for all assay methods, for the one-stage assay higher potencies were obtained if FVIII deficient plasma was used (Lee *et al*, 1983). However, a difference was not observed in the chromogenic assay, possibly due to the presence of bovine serum albumin in the buffer supplied with the chromogenic kit (Mazurier *et al*, 1990). This combined work led to recommendations by the Scientific and Standardisation Committee (SSC) of the ISTH (Barrowcliffe, 1993a) which were:

1) The use of a concentrate standard for FVIII concentrate assays
2) Test and standard should be pre-diluted in severe haemophilic plasma or artificially depleted plasma.
3) All assay buffers should contain 10mg/ml albumin
4) The chromogenic method as the reference method

These modifications improved the inter-laboratory variation in subsequent collaborative studies, and this led to the adoption of these recommendations. In a more recent collaborative study for the 6th IS, the inter-laboratory variation was 7-10% for the one-stage and 6-9% for the chromogenic assays for assays of recombinant and plasma-derived concentrates against a recombinant standard (Raut *et al*, 2001). The 6th IS was the first time that a recombinant product had been used as a standard. A subsequent study showed no differences between the use of a PD and recombinant standard (Albertengo *et al*, 2000).

1.2.7.2 Discrepancies between methods

The use of the same standard should ensure that all three FVIII assays give the same potency. However, the introduction of increasingly pure FVIII products has led to discrepancies between methods. It was first noted, more than 20 years ago in a collaborative study that there was a large disagreement between laboratories. This was thought to be mainly due to differences in reagents used. There were however still some differences when the same reagents were used. The two-stage assay detected more activity in a more highly purified freeze dried preparation that was tested against a concentrate standard (Kirkwood *et al*, 1977). A retrospective study at the same time found that the two-stage assay consistently returned values which were higher than the one-stage when concentrates were assayed against the plasma standard (Kirkwood & Barrowcliffe, 1978). Since these studies were carried out changes in assay design have occurred, so that concentrates are assayed against concentrate standards and the adoption of the other ISTH recommendations.

Adoption of the ISTH recommendations, however, has not completely overcome assay discrepancies. In a comparison of potency estimation of several concentrates by one-stage and two-stage assay, the two-stage assay gave lower results for several products (13-40% lower than one-stage). This was statistically significant for several PD products of high purity (Barrowcliffe *et al*, 1990). A more recent study between one-stage and chromogenic assays was done by Hubbard et al, in which the greatest discrepancy was observed in two monoclonal antibody purified products, in which the mean one-stage potency exceeded that

of the chromogenic potency by 33 and 24%. The BDD product was unique in this study in that the one-stage potency was 22% lower than the chromogenic (Hubbard *et al*, 2002a). It is thought that the discrepancy between the one-stage and chromogenic assays for the PD intermediate products may be due to differences in activation (Hubbard *et al*, 2002b). In a "field" study in which laboratories all analyse the same sample by their routine methods, differences were observed between the methods, these differences were less than 10 % for all concentrates apart from a monoclonal antibody purified concentrate, although this discrepancy was minimised by those laboratories which used pre-dilution in haemophilic plasma (Raut *et al*, 2003a).

The BDD product is unique in assay discrepancies in that the value obtained by the one-stage assay is lower than that of the chromogenic assay. This difference can be as much as 50% (Sandberg *et al*, 2001;Mikaelsson *et al*, 1998). However, this large assay discrepancy was found to be due to the amount and type of phospholipid used in the one-stage assay, when platelets were used in the one-stage assay discrepancies between the concentrates were eliminated (Mikaelsson *et al*, 1998).

1.2.7.3 Assays of post-infusion plasma and *in vivo* recovery

Discrepancies in the calculation of *in vivo* recovery are also a long standing problem. In 1979 Nilsson *et al* reported a 100% recovery with the APTT assay, whereas only 81% was recovered as measured by the two-stage assay when post-infusion plasmas were assayed against a plasma standard (Nilsson *et al*, 1979).

Due to the large variation between laboratories and between assay methods the assay of FVIII concentrates against a plasma standard has been avoided by the introduction of two standards. However, it is not quite so straightforward in the case of post-infusion plasma as the concentrate administered has just been diluted in the patient's plasma, and in theory may well not be equivalent to normal plasma FVIII.

Lower or higher than expected recovery levels suggest that the labelling of the FVIII concentrate may not be correct. In a retrospective study to examine this, no evidence was

found of any difference in *in vivo* recovery value between assays when concentrates were assigned potency with either a concentrate or plasma standard (Prowse, 1995).

Several pharmacokinetic studies have been performed to further address the issue of *in vivo* recovery. A pharmacokinetic study with *Hemofil-M* found large discrepancies. In this study the one-stage gave 26% higher potencies than the chromogenic on the concentrate. However, the recovery was 17-28% higher by chromogenic assay vs one-stage after correction for potency labelling (Lee *et al*, 1996a). If the concentrate potency was assigned by chromogenic and post-infusion samples were assayed by one-stage, the recovery levels were higher than predicted by 11-25%, and this is a situation that would arise routinely. In another study the same group used a recombinant product and found that significantly higher recoveries were obtained with the chromogenic assay. However, the use of *Recombinate* diluted in haemophilic plasma as the standard eliminated discrepancies between the two methods (Lee *et al*, 1999).

When concentrate potency is measured by chromogenic assay, the recovery values are higher when measured by chromogenic vs one-stage assay, which would lead to an overestimated predicted recovery in a plasma derived product (Lee *et al*, 1996b), higher recovery levels have also been demonstrated for recombinant products (Lee *et al*, 1999;Lusher *et al*, 1998).

It has been found that assay discrepancies can be eliminated when a concentrate standard is used to assay post-infusion plasma samples (Lee *et al*, 1999;Mikaelsson & Oswaldsson, 2002;Mikaelsson *et al*, 2001). Traditionally the standard used has been a like vs like i.e. a plasma standard for assays on patient samples and a concentrate standard for assays of concentrates. In the case of post-infusion samples it may be more appropriate to think of post-infusion samples as concentrate diluted in the patient's plasma, therefore a concentrate standard diluted in haemophilic plasma would be the more appropriate choice (Barrowcliffe *et al*, 1998).

A product specific laboratory standard is available for *Refacto*. In a comparison of one-stage and chromogenic values in post-infusion samples using the *Refacto* standard 9% lower values were obtained with the one-stage assay, which the authors found acceptable for assays on post-infusion plasma samples (Sukhu *et al*, 2003).

Hubbard et al investigated the differences between the assays and found that plasma FVIII behaves differently in the chromogenic assay, with more rapid thrombin generation, but slower FXa generation (Hubbard *et al*, 2001). This study supports the use of a concentrate standard.

1.2.7.4 Assays discrepancies in mild/moderate haemophilia

There is a subgroup of patients with mild haemophilia in whom discordant assay results are reported between one-stage and two-stage assays. The one-stage assay is found to give 2-7 fold more FVIII than is measured in the two-stage assay (Duncan *et al*, 1994). Several different missense mutations have been reported all occurring in the A1, A2 or A3 domains (Rudzki *et al*, 1996;Mazurier *et al*, 1997;Keeling *et al*, 1999;Schwaab *et al*, 2000). Closer investigation of one of these mutations, Arg 531 His, revealed a higher dissociation rate of the A2 subunit from the activated heterotrimer (Pipe *et al*, 1999) and this most likely accounts for the differences.

1.3 Thrombin generation test

1.3.1 Origin

The thrombin generation test (TGT) has been used for almost 50 years, and was originally developed by Macfarlane and Biggs (Macfarlane & Biggs, 1953) for use in whole blood and simultaneously by Pitney and Dacie for citrated plasma (Pitney & Dacie, 1953). Both methods were similar in that blood or plasma was placed in glass tubes with saline, subsamples were removed into a fibrinogen solution and the time taken for the fibrinogen to clot was measured.

In more recent years Hemker's group has developed this assay further. Initially this group used the chromogenic substrate S-2238 for measuring the amount of thrombin generated in each subsample. The advantage of this method was that more subsamples could be taken than the limitations involved in performing the TGT on a coagulometer or by hand. The disadvantage of using a chromogenic substrate though is that it reacts not only with thrombin but also the complex of α2-macroglobulin-thrombin. However, this can be overcome by the use of a simple mathematical procedure applied to the raw data, to obtain the amount of free thrombin. A further refinement of the chromogenic method was the use of a slow chromogenic substrate, this allowed the development of a continuous non-subsampling system in which samples could be loaded onto a microtitre plate and read at regular intervals. Again a correction needs to be made for α2-macroglobulin-thrombin complex. The disadvantage of the continuous chromogenic method is that the plasma must be optically clear i.e. plasma must be defibrinated so that the clot does not interfere with the optical density (OD) readings. The method is also unsuitable for measuring thrombin generation in platelet rich plasma PRP, which must be performed by a subsampling methodology. The most recent advances in the TGT has been the use of a fluorogenic substrate (Hemker *et al*, 2000), which is unaffected by the turbidity of the plasma and allows thrombin generation to be determined in plasma which is un-defibrinated, in PRP and also possibly in whole blood.

1.3.2 TGT in haemostasis

Although the TGT has never reached widespread use in haemophilia treatment, a chromogenic version was used to monitor the effect of DAAVP infusion in mild haemophilia (Keularts *et al*, 2000), and has been used to study FVIII-inhibitor bypassing agents (Turecek *et al*, 2003;Barrowcliffe *et al*, 1983). The TGT has been used extensively in anticoagulant studies (Hemker, 1987;Hemker & Béguin, 2000;Lawrie *et al*, 2003) and is becoming more widespread since a commercial company was established in 2002 which provides the software necessary to convert a fluorescent signal into thrombin generation in real time (Hemker *et al*, 2003).

1.3.3 Other global tests for haemostasis

The increase in use of the thrombin generation test has been mirrored by increasing use of other tests which measure global haemostasis. The first of these was the thromboelastogram (TEG), which although it has also been in use for almost 50 years, has been used principally by anaesthetists to measure the haemostatic profile during liver operations (Vig *et al*, 2001). Ingerslev et al have used the ro-TEG (which is a modified version which records the data electronically, which allows for post-test analysis) to study patients with haemophilia but have transformed the data in such a way that it resembles a thrombin generation curve, this method is now known as ROTEM [321 /id]. However, the parameters measured are different in that the first derivative is the velocity of clot formation; therefore the ROTEM only provides information about the initiation of coagulation. The ROTEM at low TF concentrations clearly shows differences between normal and those with severe haemophilia A, however, at high TF concentration the velocity of clot development was similar (Sørensen *et al*, 2003). The ROTEM has also been shown to be sensitive to FVIII levels less than 0.001 IU/ml when triggered with a low TF concentration.

He at al have developed an assay to determine the haemostatic potential of plasma (He *et al*, 2001). This assay is based on the principal of adding a small amount of thrombin and t-PA to the plasma and monitoring aggregation of fibrin at 405nm. Thrombin converts the fibrinogen to fibrin and the t-PA produces plasmin, which is then able to break down the fibrin. The area under the fibrin curve represents the overall haemostatic potential (OHP). In this assay, a commercial FVIII deficient plasma did not generate a detectable OHP (He *et al*, 2001). With inclusion of platelets in the system FVIII <1% generated an OHP of less than the 2.5 percentile of the normal reference range, FVIII of 1-5 and 6-15% was distinguishable from the FVIII deficient plasma, and at 30% FVIII the OHP entered into the fifth percentile which was classified as a normal OHP (Antovic *et al*, 2003). This method is also applicable to measurement of hypercoaguable situations and monitoring of heparin treatment (Antovic *et al*, 2002).

Calatzis et al have developed a thrombin dynamics test (TDT) in which the dynamics of the initial thrombin formation is assessed. This method was able to distinguish between different FVIII levels (Calatzis *et al*, 2003).

Carr et al developed a test for measuring the thrombin generation time, which is measured from the platelet contractile force. Both FVIII and FIX deficiency resulted in a prolonged thrombin generation time. Replacement of FVIII decreased the thrombin generation time to normal (Carr *et al*, 2003).

The PT and APTT clotting times are detected on automated coagulometers by changes in the light transmittance, and from this other parameters can be obtained to give more detailed information on formation of the clot. The raw data can currently only be detected from one machine, the MDA 180 by Organon Teknika (Braun *et al*, 1997). When clotting is measured by the APTT a biphasic waveform occurs in patients with disseminated intravascular coagulation (DIC) (Downey *et al*, 1997). This is particularly valuable as the changes in the waveform are apparent before the standard laboratory tests can indicate DIC (Downey *et al*, 1998;Toh *et al*, 2002). An abnormal waveform has also been observed in patients with anti-phospholipid antibodies when the PT is measured (Su *et al*, 2002). This technique has also been applied to haemophilia and was able to differentiate between FVIII concentrations down to 0.002 IU/ml (Shima *et al*, 2002).

1.4 Aims

The aim of this study was to use a more physiological approach to the TGT to investigate assay discrepancies. This was to be by examination of thrombin generation profiles of various FVIII concentrates of differing purities under various assay conditions, including the use of platelets. In addition differences between concentrates were also to be examined using a purified FXa generating assay. It was also planned to use the TGT to examine thrombin generation in haemophilic plasmas, to see if this assay would be more sensitive than currently used assays, and also to examine thrombin generation profiles at trough FVIII levels in a group of patients on prophylaxis.

CHAPTER 2
MATERIALS AND METHODS

2.1 Plasma preparation

Haemophilic plasmas

Blood was collected into citrated tubes (Tri-sodium citrate 0.106 M). Blood was spun at 3000g for 15minutes at 4°C. Plasma was removed and the centrifugation repeated. Plasma was kept frozen in aliquots at -80°C.

Normal pooled plasma

Seven units of normal plasma were obtained from the National Blood Service (Colindale, London). These were collected by conventional venepuncture into citrate phosphate dextrose-adenine (CPD-A) anticoagulant, containing 0.106 M total citrate, at a ratio of 6ml CPD-A to 450ml whole blood. Each unit was centrifuged twice, and was buffered by the addition of HEPES, final concentration 20mM. The units were pooled and frozen in aliquots at -80°C.

2.2 FVIII assays

2.2.1 One-stage APTT assay

Reagents

Glyoxaline:	50mM Imidazole, 100mM NaCl, 0.02% sodium azide, 1 % (v/v) HSA (Zenalb, BPL, Elstree, Herts)
APTT reagent:	IL (Warrington, UK)
FVIII deficient plasma:	Technoclone (Dorking, Surrey)
Deca coagulometer	Grifols (Barcelona, Spain)
FVIII standards	20th British Standard for plasma 6th IS for FVIII concentrates

Method

A standard and all test samples were diluted by 1in 10, 1in 30 and 1 in 100 in glyoxaline buffer. Samples were assayed in a reverse balanced order. 100µl of APTT reagent, 100µl of FVIII deficient plasma and 100µl of test or standard dilution were warmed at 37°C for 5 minutes on a Deca coagulometer. 100µl of Ca^{2+} was then added and the time taken for the mixture to clot was recorded by the Deca coagulometer, which detected clotting by means of a mechanical end point.

The FVIII concentration in the samples was obtained by use of a parallel line software package (NIBSC in-house software), comparing the unknown FVIII concentration in the test sample to the known amount of FVIII in the standard.

2.2.2 APTT blank time

100µl APTT lyophilised silica, 100µl FVIII deficient plasma, 100µl glyoxaline buffer was incubated for 5 minutes at 37°C on Deca coagulometers, at which point 100µl Ca^{2+} was added and the time taken for the clot to form was recorded. Each plasma was measured in duplicate in a reverse balanced order.

2.2.3 Chromogenic FVIII assay

Reagents

Coatest C/4 kit	Chromogenix (Quadratech, Epsom, Surrey)
FVIII deficient plasma	Organon (Biomerieux, Basingstoke, Hampshire)

Method

FVIII was measured using a Coatest C/4 kit. The manufacture's instructions were followed. FX/FIXa, PL and substrate were reconstituted. The dilution buffer was made by diluting stock buffer 1 in 10 with water containing 1% HSA.

FVIII concentrates and standards were diluted to 6 IU/ml in FVIII deficient plasma, they were then further diluted to 1 IU/ml in buffer. Test dilutions were then made in replicate with buffer to 1 in 50, 1in 100 and 1 in 200. Samples were loaded in a reverse balanced

order on the ACL 3000 (IL) and the kit reagents added as required. Plasma samples were treated as above apart from no predilution and use of a plasma standard. The ACL 3000 was set up in the following manner: 25µl of sample was incubated with 75µl of combined reagent for 300 seconds, subsequently 50µl of chromogenic substrate was added, OD at 405nm was measured following a further 220 second incubation period.
FVIII concentration was established by parallel-line bioassay as describe in 2.1.1, using OD values instead of clotting times

2.2.4 FVIII:Ag binding assay (ELISA)

Reagents

Glycine buffered saline (GBS) pH 9.2:	20mM glycine, 34mM NaCl
Phosphate buffered saline (PBS) pH 7.4:	20mM Na_2HPO_4, 67mM KCl, 4.4mM KH_2PO_4, 140mM NaCl
PBS-Tween (PBS-T):	PBS with 0.05% (v/v) Tween 20
PBS/HSA	PBS with 5% (v/v) human serum albumin
Orthophenylenediamine (OPD)	Dako (Ely, Cambridgeshire)
Microtitre plate	Maxisorp (Nunc, Sigma, Pool, UK)

Method

A 96-well microtitre plate was coated with 100µl of a monoclonal anti-FVIII Ab F7B4 (kind gift of Dr Saint-Remy) at a dilution of 1 in 1000 in GBS and incubated overnight at 4°C. The plate was then washed 4 times with PBS-T and blocked with PBS containing 5% HSA for 1 hr at room temperature (RT). The plate was then washed again 3 times with PBS-T. FVIII for the standard curve was diluted in PBS-T and 100µl was added to the plate, samples were added to the plate also and incubated for 2hours at RT. The plate was once again washed 3 times with PBS-T and 100µl of a rabbit polyclonal anti-FVIII Ab (Kind gift from Dr Ingerslev) was added at a dilution of 1in 2000 in PBS-T and incubated for 1 hr at RT. The plate was washed and the Tag antibody horseradish peroxidase-conjugated Goat Anti-Rabbit immunoglobulins (DAKO) 100µl was added at 1in 2000 in PBS-T for 1hr at RT. After a final washing, 100µl of OPD substrate was added and the colour allowed to develop for 30minutes before termination of the reaction with 100µl

0.5M sulphuric acid. The plate was read at 490nm in a Spectramax 250 plate reader (Molecular Devices, Wokingham, Berkshire).

2.3 Thrombin generation tests

2.3.1 Clotting method

Reagents

Tris buffered saline (TBS):	50mM Tris, 150mM NaCl, 0.02% NaN_2
TBS/HSA:	TBS containing 1%(v/v) human serum albumin
Saline:	154mM NaCl
Fibrinogen	Diagen bovine fibrinogen (Diagnostic Reagents, Thame, Oxon)
FIXa	11 IU/ml/ 834nM NIBSC (97/572). Derived from recombinant FIX, which was then activated with FXIa. Ampoules reconstituted with water and snap frozen.
TF	RecombiPlasTin (IL). Batches were calibrated by Steve Thomas using a protrhombin time assay against NIBSC preparation 94/738, which had previously been calibrated against American Diagnostica Lipidated Recombinant Tissue Factor (Product 4500L, Greenwich, CT, USA). TF was reconstituted with 10mls water and used within two weeks.
PL	Derived from bovine brain, consists of PC 24.7%, PE 38.9%, PS 28.5%, PI 5.7%, SM 2.2% (as analysed by HPLC using Folch extracts by Bill Pickering). 10mg/ml NIBSC (91/542), ampoule reconstituted in water and aliquots snap frozen
Thrombin	100 IU/ml NIBSC (89/588)
FVIII deficient plasmas	Organon (Biomerieux, Basingstoke, Hampshire) Dade-Behring (Sysmex, Milton Keynes) Technoclone (Dorking, Surrey) Sigma (Poole, UK)

Defibrination of plasma

Plasma was defibrinated with ancrod (55 IU/ml 74/581) final concentration 0.5 IU/ml for 30 minutes at 37°C. The clot was then removed by winding onto a wooden applicator stick. If plasma was not defibrinated before use in the TGT then the clot would develop during the course of the assay, at which point the clot was wound onto a wooden stick and all plasma squeezed out of the clot which was then left in the reaction.

Method

400µl plasma was incubated with 80µl FIXa (diluted 1 in 10) for 90 seconds at 37°C, after which 400µl PL (10µg/ml) was then added. Finally 400µl $CaCl_2$ (25mM) was added at 2 minutes to initiate thrombin generation. Reaction mixture was mixed and placed in a pre-warmed Eppendorf repeator pipette, at timed intervals of between 15-60 seconds 50µl was added to 200µl of fibrinogen, which was contained in a rotating cup on a Deca machine. The time taken for the clot to form was measured for 18 subsamples on two machines. The amount of thrombin in each subsample was calculated from a thrombin standard curve, which was made from a range of thrombin 50-0.39 IU/ml.

Data analysis

To calculate the amount of thrombin generated at each time point clotting times were inputted into "tap" which was an in-house (NIBSC) programme. Tap calculated thrombin in each sample from a thrombin standard curve, the programme plotted each point on a graph and calculated area under the curve (AUC). Peak-height was determined from the transformed data. Time-to-reach-half-peak-heigh ($T_{½max}$) was determined by reading from the graph the point at which half the peak height had been generated.

2.3.2 Fluorogenic method

Reagents

A 10mM solution of Z-gly-gly-arg-AMC (Bachem, St Helens, UK) was prepared in 20mM Hepes, 150mM NaCl, 60mg/ml bovine serum albumin (BSA, Sigma) and 10% (v/v) DMSO. This solution was aliquoted and stored at –40°C.

Method

40µl of un-defibrinated plasmas were loaded onto a black microtitre plate (Greiner, Sigma). The substrate mixture was prepared by combining all remaining reagents, 1 part substrate 10mM: 2.55 parts FIXa (97/572, diluted 1 in 10): 11.72 parts Ca^{2+} (25mM): 12.76 parts PL (91/542, 10µg/ml). The mixture was warmed at 37°C and 80µl was added to each well on the microtitre plate. In each well the plasma was diluted by two-thirds, and the final concentrations were: substrate 0.238mM, FIXa 5nM, Ca^{2+} 7mM, PL 3µg/ml. The plate was placed in a Spectramax Gemini XS (Molecular Devices) at 37°C. The plate was read at 30 seconds intervals for 1 hr, with excitation at 390nm and reading at 460nm.

Data analysis

The data was exported into an excel file and the amount of thrombin generated was calculated according to the method by Hemker (Hemker & Béguin, 1999) (Table 2.1, Fig 2.1). This method was used by Hemker et al to determine the amount of free thrombin in a continuous TGT with a chromogenic substrate, and also with a fluorogenic substrate (Hemker *et al*, 2000). However, recently this group has developed another method for determination of thrombin in the fluorogenic TGT (Hemker *et al*, 2003).

Table 2.1 Calculation of free thrombin from relative fluorogenic units. Subsampling times are entered into column B and corresponding RFU values are entered into column C. In column E the amount of product due to α2-macrogloubulin-thrombin activity is calculated. In column D the amount of product due to the activity of free thrombin is calculated using an assumed value of k (cell $F$1).

	A	B	C	D	E	F
1		Time	Data	Product from thrombin	Product from α2-macroglobulin	k
2		0.5	0	0	0	=sum(F112:F122)
3		1		=C3-E2	=E2+($F$1*D3)	
4		1.5		=C4-E3	=E3+($F$1*D4)	=(D4-D3)/0.5
5		2		=C5-E4	=E4+($F$1*D5)	=(D5-D4)/0.5
.						
121		59.5		=C121-E120	=E120+($F$1*D121)	=(D121-D120)/0.5
122		60		=C122-E121	=E121+($F$1*D122)	=(D122-D121)/0.5

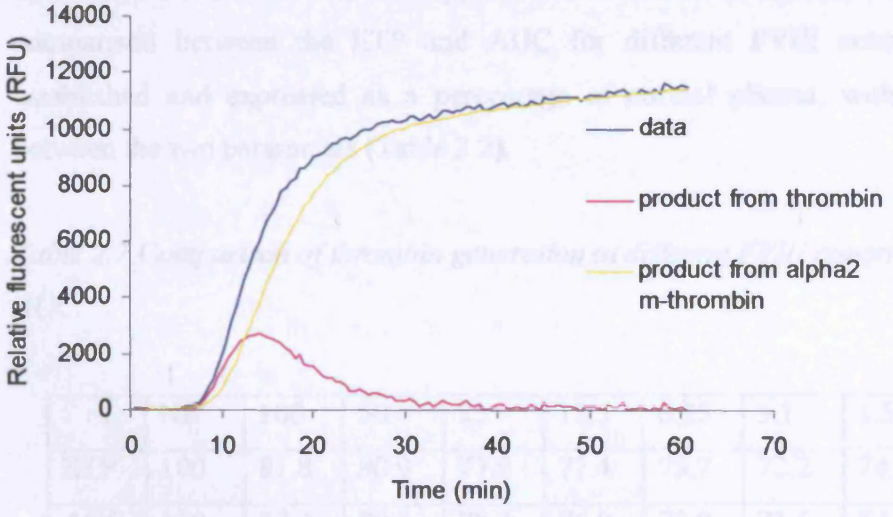


Fig 2.1 Calculation of free thrombin

Cell F2 is the sum of the last ten derivateives of thrombin generation in column F, this value should be zero as free thrombin is assumed to have reached the zero level. The value of K (Table 2.1) is set such that the sum (cell F2) is equal to, or close to zero. However, in practice this was difficult to achieve due to the large variation in readings at the plateau.

This is the calculation that is performed in order to calculate the contribution by the α2-macroglobulin which also combines with chromogenic and fluorogenic substrates. This data calculation was developed by Hemker et al for the chromogenic substrate, but has also been used by them for fluorogenic calculation. However, I have adapted this slightly for use with the fluorogenic system as the method for determination of k did not work on a practical level, due to the large variation in values at the plateau phase due to the large number of units, so that a value close to zero was not possible in a vast number of cases, and even with the same plasma source the values of k varied widely. In order to achieve comparable results k was set to 0.155 for all assays. This allowed a thrombin generation curve to be obtained for all plasmas used and at all FVIII concentrations tested. Using k at 0.155 there was a similar CV between the final fluorogenic reading which is also a marker for endogenous thrombin potential (ETP) and the AUC, which after transformation of the data was 4.8 and 4.6% respectively, this was measured for normal plasma in 48 wells. A comparison between the ETP and AUC for different FVIII concentrations was also established and expressed as a percentage of normal plasma, with comparable results between the two parameters (Table 2.2).

Table 2.2 Comparison of thrombin generation at different FVIII concentrations by ETP and AUC.

	NP	100	50	25	12.5	6.25	3.1	1.58	0.78	BL
ETP	100	81.8	80.9	77.9	77.4	73.7	72.2	74.8	72.0	57.2
AUC	100	82.2	80.4	78.4	76.8	73.9	71.6	74.3	72.0	57.0

A macro was established to calculate free thrombin. From the free-thrombin data time-to-peak and peak-height were established (Table 2.3). In addition AUC was calculated by the trapezium rule.

Table 2.3 Equations for the calculation of peak height and time to peak from free thrombin values.

	A	B	C	D
1		1	2	3
2	Time to peak	=INDEX($A$6:$A$125,B3,1)	=INDEX($A$6:$A$125,C3,1)	=INDEX($A$6:$A$125,D3,1)
3		=MATCH(B4,B6:B125,0)	=MATCH(C4,C6:C125,0)	=MATCH(D4,D6:D125,0)
4	Peak height	=MAX(B6:B125)	=MAX(C6:C125)	=MAX(D6:D125)
5		1	2	3
6	1	Data	Data	Data
7	1.5	Data	Data	Data

2.4 Factor Xa generation assay

Reagents

Tris/EDTA	175mM NaCl, 50mM Tris, 7.5mM EDTA pH 8.4
S-2765	Chromogenix (Quadratech)
Human FXa	Haematologic Technologies (Pinewood Plaza, VT, USA)

Method

100µl of EDTA buffer was added to each well of a 96 well flat-bottomed micro-titre plate (Greiner). 300µl hirudin (Revasc, 23.3µg/ml), 300µl phospholipid (91/542, 50µg/ml), 300µl FX (98/574 1 IU/ml), 300µl FIXa (97/562, 0.4 IU/ml) were heated together for 5 minutes at 37°C. Heated separately were FVIII (6 IU/ml) and $CaCl_2$ (25mM). At the end of incubation time, 300µl $CaCl_2$ was added along with 300µl FVIII and reaction started. At timed intervals 50µl subsamples were removed into the EDTA buffer which stop any further generation of FXa. In some experiments FVIII was activated with thrombin (89/588, NIBSC), FVIII was incubated wth thrombin, at the end of this step further activation of FVIII was terminated by addition of hirudin, the remaining reagents were then added. Once all subsamples were obtained, 50µl 1.5mM S2765 was added to each well, after 3 minutes the colour developing reaction was terminated with 50µl 50% acetic acid.

The plate was read at 405nm, OD values were converted into FXa concentration using a FXa standard prepared with Human FXa.

2.5 Platelet Isolation

Blood collection

Acid citrated dextrose-A (ACD-A) 70mM sodium citrate, 40mM citric acid, 120mM glucose, pH 5.0).

Blood was collected from healthy volunteers that had not consumed any non-steroidal anti-inflammatory agent for at least 7 days prior. Blood was taken by venipuncture from the antecubital vein. 8.5 parts of blood was drawn into 1.5 parts into the citrate anti-coagulant ACD-A.

Platelet isolation

Whole blood was spun at 150g for 10 minutes and the PRP was removed. PRP was mixed with 1/10 the PRP volume of 1mM adenosine and incubated for 5 minutes at RT, 1/10 the volume of ACD-A was then added.

Albumin density gradient

A 50% albumin solution was prepared by adding 5 g of bovine serum albumin (Sigma, A7906) to 10 mls of water and allowed to dissolve undisturbed at 4°C. 2mls of 50% albumin was placed at the bottom of a centrifuge tube. Layered on top of this was 1 ml each of 25, 17, 12 and 10% solutions diluted in Hepes buffer.

3 mls of the PRP prepared above was then layered onto the albumin density gradient and spun at 650g for 15 minutes. The plasma and some of the albumin was removed, the concentrated platelets at the interface of the 50% layer were carefully removed along with some of the albumin.

Gel filtration

HEPES buffer- 137mM NaCl, 2.7mM KCl, 1mM $MgCl_2$, 5.5mM glucose, 0.35% BSA, 3mM NaH_2PO_4 and 3.5mM HEPES, pH 7.3.

Preparation of column

Column was made up of a 60 ml monoject syringe for the column and at the bottom of the syringe a 25mm diameter stainless steel filter support (Millipore, XX3002510) on top of which was placed a 40 nm nylon mesh filter (25mm diameter) which was held in place by an O-ring. Sepharose 2B (Amersham) was washed with 3 volumes of saline to remove storage ethanol, and then suspended in saline. A small volume of sepharose 2B was pippetted into the bottom of the capped syringe, carefully avoiding air bubbles between the filter and the sepharose. More sepharose was transferred into the column until 20mls was reached, at which point the syringe was uncapped and the buffer allowed to flow through the column, more sepharose was added until the final bed height was 50 mls. The packed column was attached to a gravifax machine and washed through with 3 volumes of saline at a flow rate of 1ml/min. Once prepared the column was stored at 4°C. Before each use the column was brought to room temperature and then equibrilated with one bed volume of Hepes buffer with glucose and albumin. After each use the column was washed through with 2 volumes of saline.

The albumin containing platelets was then carefully applied to the top of the column with a plastic Pasteur pipette. The platelets were then eluted and collected in 1ml fractions with Hepes buffer containing glucose and albumin at a rate of 1ml/min. All platelet fractions were pooled together and 1/10 of the volume of ACD-A was added, platelets were spun at 1000g for 5 minutes, the supernatant was removed and the platelets resuspended in the residual buffer. Platelets were allowed to recover for 30 minutes at room temperature before being used in thrombin generation assay.

CHAPTER 3
STUDIES OF FVIII CONCENTRATES

3.1 Introduction

Discrepancies between chromogenic and one-stage APTT assays have led to an uncertainty about the true FVIII level. An accurate FVIII level is critical in the management of haemophilia and should reflect the haemostatic effectiveness of FVIII concentrates so that patients are neither under or over-treated. We have thus applied a more physiological approach by using the TGT to assess the potential haemostatic effectiveness of different FVIII therapeutic concentrates.

This chapter describes the studies of a wide range of FVIII concentrates using the clotting TGT and a FXa generating assay.

3.2 Initial investigations

The TGT method at NIBSC was established by Barrowcliffe et al in which one part of plasma was incubated with one part PL and one part Ca^{2+} in glass tubes, subsamples were then taken into fibrinogen (Barrowcliffe *et al*, 1983) . A further modification was made to standardise activation with FIXa instead of glass (Barrowcliffe *et al*, 2002). In this study, to optimise the TGT for measurement of FVIII concentrates a number of further modifications were made. These included addition of various commercial FVIII deficient plasmas and investigation of optimal PL concentration, each one described below.

3.2.1 Choice of FVIII deficient plasma

A FVIII deficient plasma was required which would give minimal thrombin generation on its own, so that all thrombin generation would be due to the FVIII concentrate alone.

Three different FVIII deficient plasmas were examined: Dade-Behring, Immunads and Organon. Dade-Behring and Technoclone plasma were depleted by FVIII antibodies, whereas Organon was depleted by a chemical method, all plasmas contained a normal

VWF level. Plasmas were first defibrinated by incubation with ancrod for 30 minutes at 37°C. Ancrod is a snake venom from the Malayan viper (*Calloselasma rhodostoma*) which acts as a fibrinogen activator, acting on the α-chain, it however, has no effect on FXIII (Hutton & Warrell, 1993). Thrombin generation was measured in duplicate on three occasions by incubating 400µl defibrinated plasma with 80µl FIXa (84nM), 400µl PL (50µg/ml) and 400µl Ca^{2+} (25mM). All the FVIII deficient plasmas produced some thrombin (Fig 3.1). The plasma from Dade-Behring produced the most thrombin with an AUC of 2156.1±690.5 IU.seconds, Technoclone produced 1496.3±378.2 IU.seconds and Organon formed the least amount of thrombin, AUC 1358.3±682.5 IU.seconds. Although these differences were not significant, due to the large variation in these experiments, Organon was chosen for subsequent use.

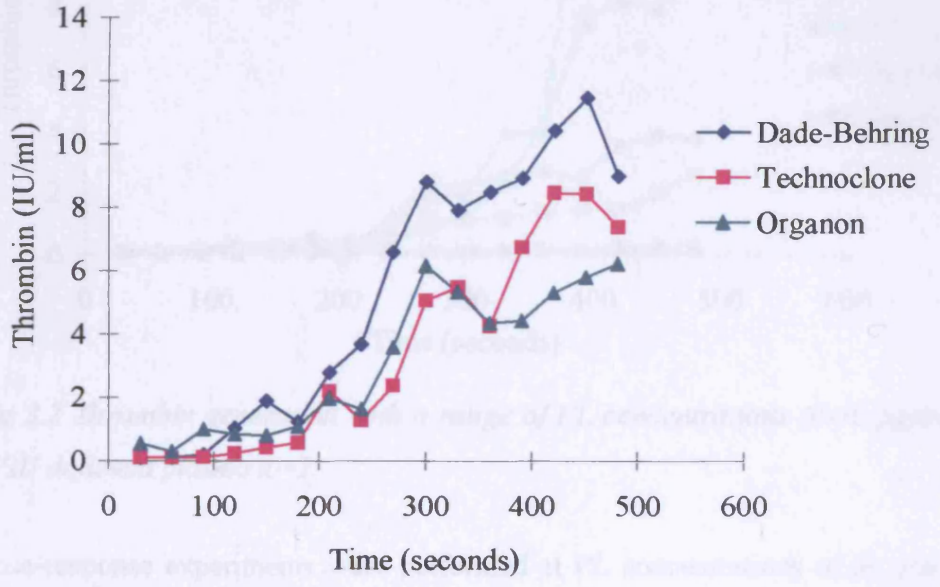


Fig 3.1 Thrombin generation by three commercial FVIII deficient plasmas.

3.2.2 Phospholipid concentration

The next step was to establish the optimal concentration of PL. A concentration of PL that would give minimal thrombin generation by the FVIII deficient plasma alone and maximal separation between different FVIII concentrations was desired, so that if there were any differences between FVIII concentrates, they would be detected.

Thrombin generation was measured in Organon FVIII deficient plasma with PL concentrations of between 50 and 5µg/ml (Fig 3.2) in duplicate samples. There was almost no thrombin generation with the very low concentration of PL (5 µg/ml) and in the absence of PL. However, a considerable amount of thrombin was generated at 10 and 7.5µg/ml, and was greater than that generated by higher concentrations of PL 50 and 25 µg/ml. This suggested that the optimal concentration for thrombin generation was between 5-10 µg/ml.

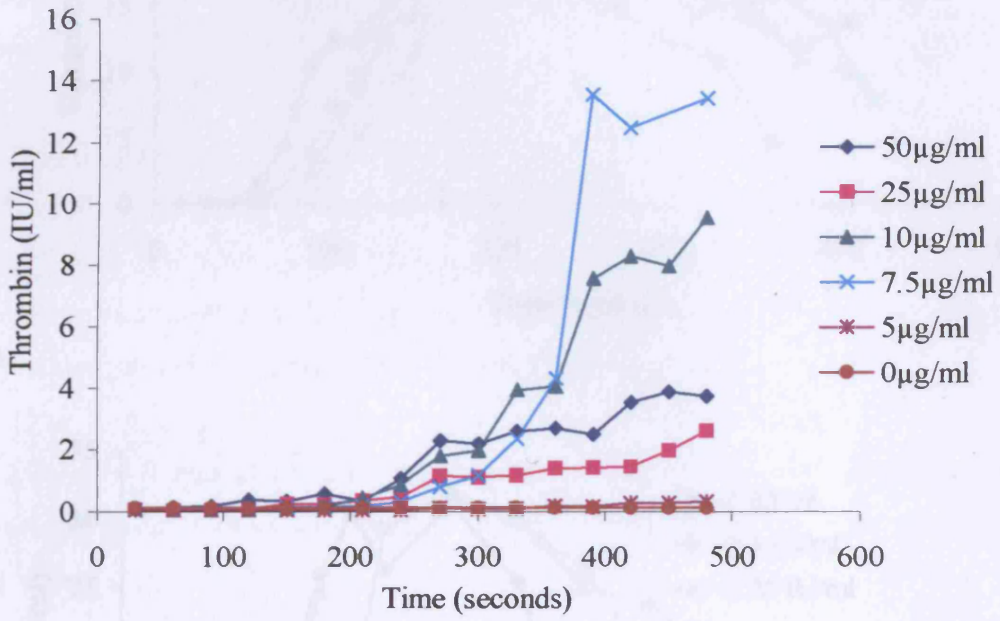
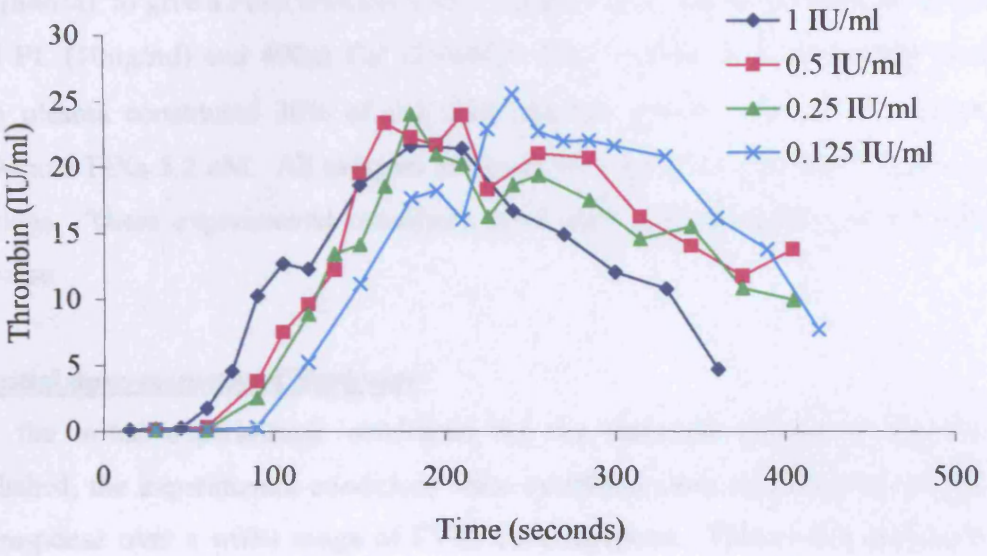


Fig 3.2 Thrombin generation with a range of PL concentrations (0-50 µg/ml) in Organon FVIII deficient plasma n=2.

Dose-response experiments were performed at PL concentrations of 5µg/ml and 10µg/ml using *Replenate* (a FVIII concentrate). *Replenate* was added to the FVIII deficient plasma to give a FVIII concentration in plasma of 1, 0.5, 0.25 and 0.125 IU/ml. The thrombin generation profiles were very similar and there was no discernable difference between 0.5 and 0.25 IU/ml FVIII, $T_{½max}$ 134 and 129 seconds respectively (Fig 3.3 A). Dose-response experiments were repeated for other concentrations of PL. At a PL concentration of 10µg/ml there was maximal separation between FVIII concentrations (Fig 3.3 B), as with a difference between 0.5 and 0.25 IU/ml FVIII of $T_{½max}$ 79 and 94 seconds respectively. 10µg/ml of PL was selected for subsequent experiments.

A



B

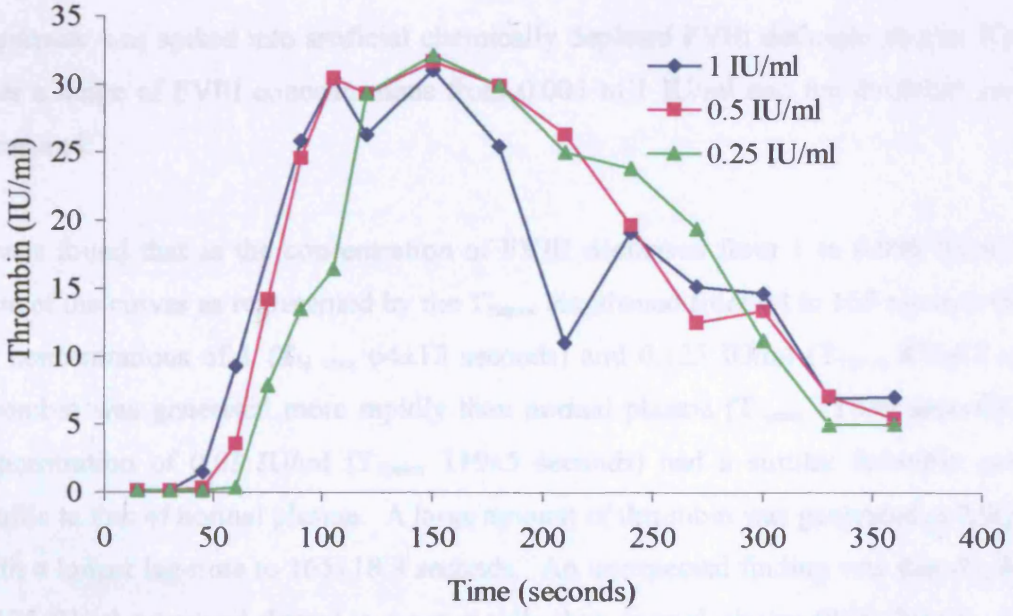


Fig 3.3 Dose-response of thrombin generation to FVIII with two concentrations of PL. A) PL 5µg/ml, B) PL 10µg/ml., n=1

3.2.3 Summary of TGT conditions

The final experimental conditions were to activate 400µl defibrinated plasma with 80µl FIXa (84nM), to give a FIXa concentration in plasma of 14nM for 90 seconds, followed by 400µl PL (10µg/ml) and 400µl Ca^{2+}(25mM). This resulted in final reaction mixture in which plasma constituted 30% of the final reaction volume, with PL 3.1 µg/ml, Ca^{2+} 7.8mM and FIXa 5.2 nM. All samples analysed were tested in duplicate on three separate occasions. These experimental conditions have been used throughout unless mentioned otherwise.

3.3 Initial dose-response of *Replenate*

Once the initial experimental conditions for the thrombin generation test had been established, the experimental conditions were examined more rigorously by performing a dose-response over a wider range of FVIII concentrations. These were compared to the thrombin generation profile of pooled normal plasma (NP).

Replenate was spiked into artificial chemically depleted FVIII deficient plasma (Organon) over a range of FVIII concentrations from 0.005 to 1 IU/ml and the thrombin generation measured.

It was found that as the concentration of FVIII decreased from 1 to 0.005 IU/ml the lag time of the curves as represented by the $T_{½max}$ lengthened from 64 to 165 seconds (Fig 3.4). At concentrations of 1 ($T_{½\ max}$ 64±15 seconds) and 0.125 IU/ml ($T_{½max}$ 87±4.2 seconds) thrombin was generated more rapidly than normal plasma ($T_{½max}$ 118±9 seconds), and a concentration of 0.03 IU/ml ($T_{½max}$ 119±5 seconds) had a similar thrombin generation profile to that of normal plasma. A large amount of thrombin was generated at 0.005 IU/ml with a longer lag-time to 165±18.8 seconds. An unexpected finding was that *Replenate* at 0.125 IU/ml generated thrombin more rapidly than normal plasma (0.86 IU/ml). The lag-time with the concentrate was only comparable to normal plasma at a concentration of 0.03 IU/ml. This will be discussed more fully in section 3.6.

However, in the same experiments, peak thrombin was unaffected, and only a small decrease in the AUC was observed. Even at a FVIII concentration of 0.005 IU/ml the AUC was only slightly lower than that of normal plasma, 6540±334 and 6728±328 IU.seconds respectively.

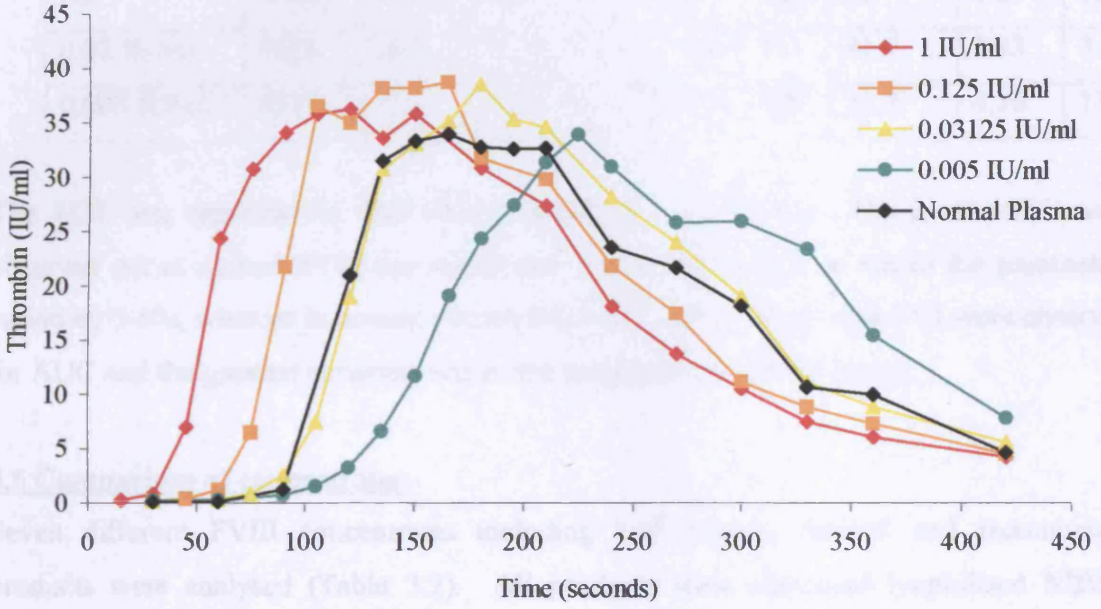


Fig 3.4 Thrombin generation by Replenate at FVIII concentrations 0.005-1 IU/ml. Replente was added to Organon FVIII deficient plasma.

3.4 Reproducibility of TGT

The reproducibility of thrombin generation was measured by repeated measurement of normal pooled plasma, and deficient plasma (Technoclone) with the addition of *Replenate* at 0.02 and 0.005 IU/ml. Thrombin generation was performed as stated above. The assays were run in duplicate on 5 different days (Table 3.1)

Table 3.1 Reproducibility of TGT parameters. Normal plasma, and Replenate added to Technoclone FVIII deficient plasma, n =5.

	AUC (IU/ml.seconds)		%	$T_{½\ max}$ (seconds)		%	Peak (IU/ml)		%
	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
NP	6069	410.5	6.8	122.5	13.1	10.7	37	4.6	12.3
0.02 IU/ml	6916	347	5	262.4	13.8	5.3	41.9	2.33	5.6
0.005 IU/ml	6372	586	9.2	361	35.4	9.8	35.7	4.16	11.7

The TGT was reproducible with CVs of between 5 and 12.3%. The lowest CVs were observed not at normal FVIII concentrations, but at 0.02 IU/ml, in which the parameters varied by 5-6%, whereas in normal plasma this was 7-12%. The lowest CVs were observed for AUC and the greatest variation was in the measurement of peak height.

3.5 Comparison of concentrates

Seven different FVIII concentrates including both plasma derived and recombinant products were analysed (Table 3.2). All products were ampouled lyophilised NIBSC preparations, an ampoules was reconstituted with 1ml water for each experiment. The experimental conditions used to measure TG were as described in 3.3 above with Organon FVIII deficient plasma.

All concentrate potencies were assigned by chromogenic assay. All concentrates at 1 IU/ml produced thrombin more rapidly, and in greater quantity, than normal plasma. The $T_{½\ max}$ of *Refacto* (48±8 seconds) was shorter than that of the other concentrates (range 54±4-64±15 seconds); this difference was statistically significant, $p<0.05$ (Fig 3.5, Table 3.3).

There was no difference between the concentrates in terms of peak height or AUC. Therefore all the concentrates were capable of generating the same amount of thrombin with the only difference being in the time at which this occurred with *Refacto* being the most rapid.

Table 3.2 Details of FVIII concentrates used in this study

	Concentrate	NIBSC code	Fractionation	VWF
Recombinant				
Refacto	B domain deleted	99/694	Recombinant	None
Recombinate	Full length recombinant	96/598	Recombinant, ion exchange and immuno affinity chromatography	None
Kogenate	Full length recombinant	96/590	Recombinant, ion exchange and immuno affinity chromatography	None
Plasma derived				
Replenate	High purity monoclonal antibody purified	96/574	Monoclonal antibody affinity chromatography	VWF:Ag <FVIII:C
Hemofil-M	High purity monoclonal antibody purified	95/640	Monoclonal antibody affinity chromatography	VWF:Ag <FVIII:C
Octavi	High purity ion-exchange	96/600	Ion exchange chromatography	Contains VWF:Ag>FVIII:C
8Y	Intermediate purity	96/576	Heparin/glycine precipitate	Contains VWF:Ag>FVIII:C

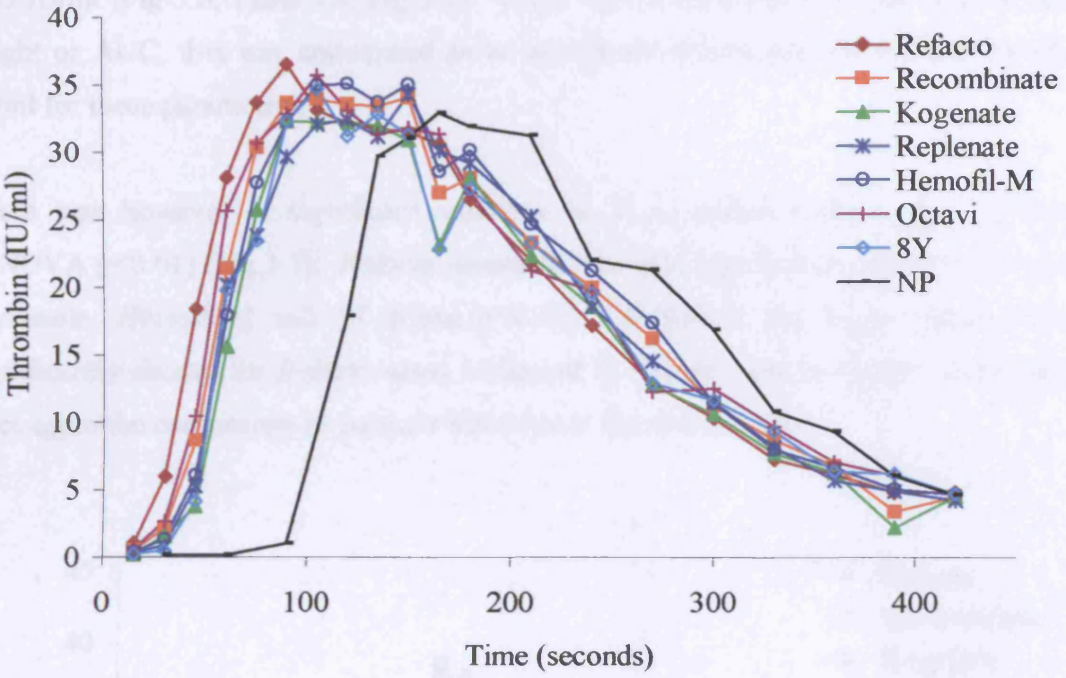


Fig 3.5 Thrombin generation by FVIII concentrates at 1 IU/ml. FVIII concentrates were added to Organon FVIII deficient plasma.

*Table 3.3. Thrombin generation parameters of FVIII concentrates at 1 IU/ml. *=p<0.05*

	AUC (IU.seconds)	$T_{½max}$ (seconds)	Peak (IU/ml)
Refacto	7888±758	48±8*	40±2
Recombinate	7514±649	57±8	39±2
Kogenate	7519±1496	64±6	38±3
Replenate	7789±997	63±8	40±3
Hemofil-M	7195±811	64±15	38±3
Octavi	7700±799	54±4	41±2
8Y	7246±815	64±9	40±2
NP	6174±735	118±9	38±3

The same seven FVIII concentrates were then measured at a lower FVIII concentration of 0.03 IU/ml (Fig 3.6, Table 3.4, Fig 3.7). There were no differences found in terms of peak height or AUC, this was anticipated as no significant differences had been observed at 1 IU/ml for these parameters.

There was however, a significant variation in $T_{½max}$ values between the concentrates (ANOVA $p<0.01$) (Fig 3.7). *Refacto* generated thrombin significantly faster than *Kogenate*, *Replenate*, *Hemofil-M* and *8Y* (t-test $p<0.05$). However, the $T_{½max}$ values were not significantly shorter for *Refacto* when compared to *Recombinate* or *Octavi*. *Kogenate* was once again the concentrate to generate thrombin at the slowest rate.

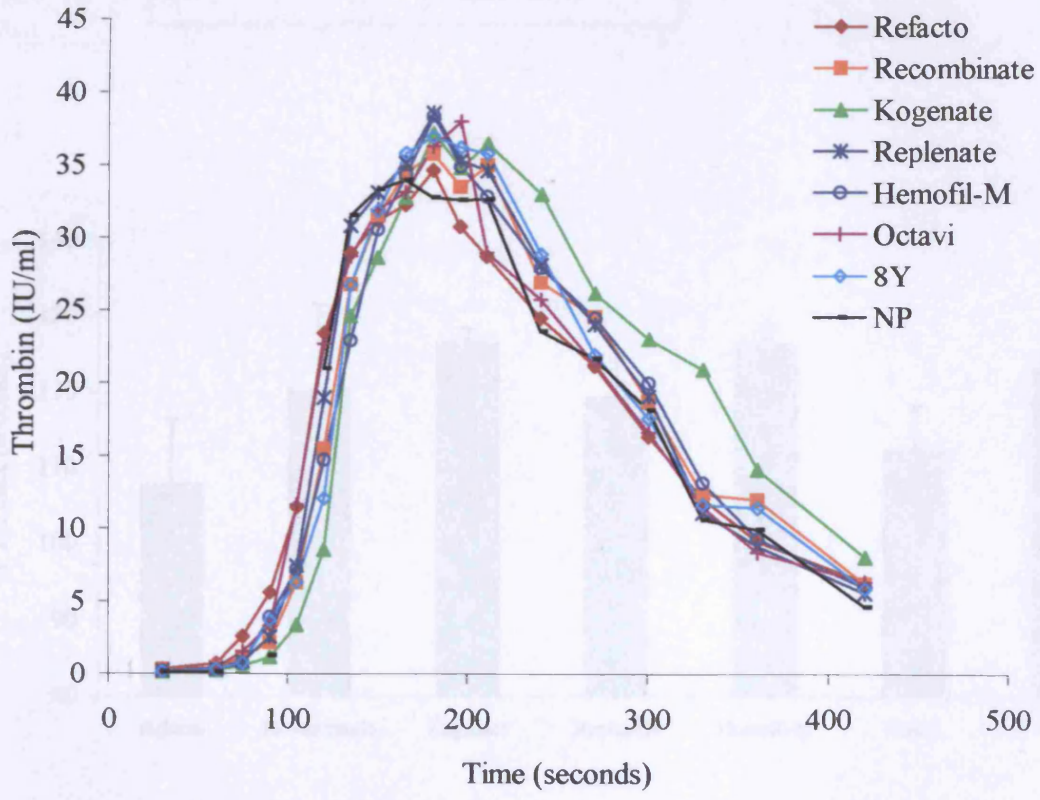


Fig 3.6 Thrombin generation by FVIII concentrates at 0.03 IU/m FVIII..

Table 3.4 $T_{½max}$ valuesof thrombin generation for FVIII concentrates at 0.03 IU/ml FVIII.

	T ½ max (seconds)
Refacto	107.7 ±8.6
Recombinate	120.0 ±11.4
Kogenate	126.3 ±1.9
Replenate	119.2 ±5.0
Hemofil-M	126.0±12.8
Octavi	112.2 ±5.8
8Y	122.6 ±9.79

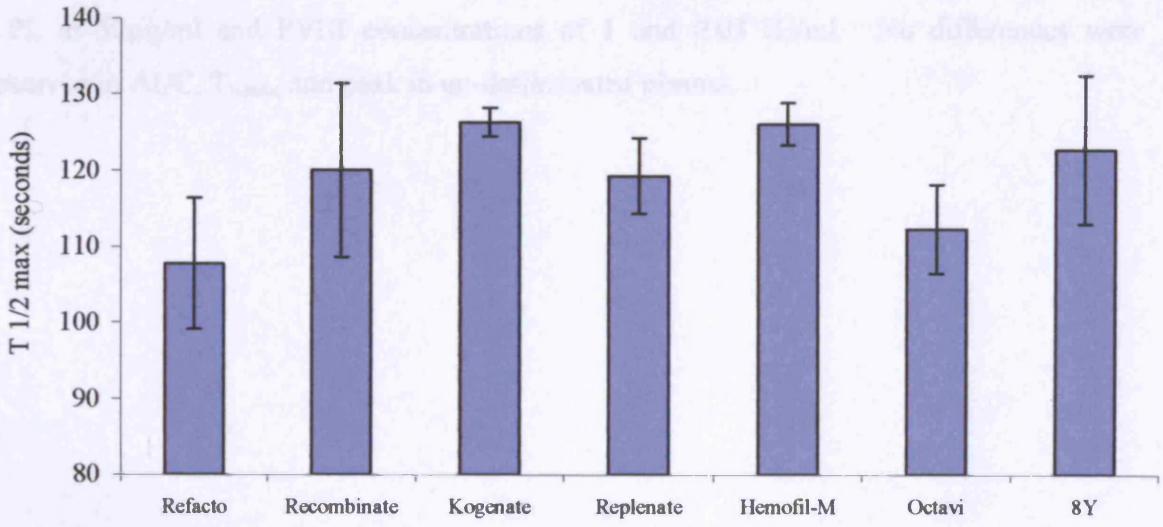


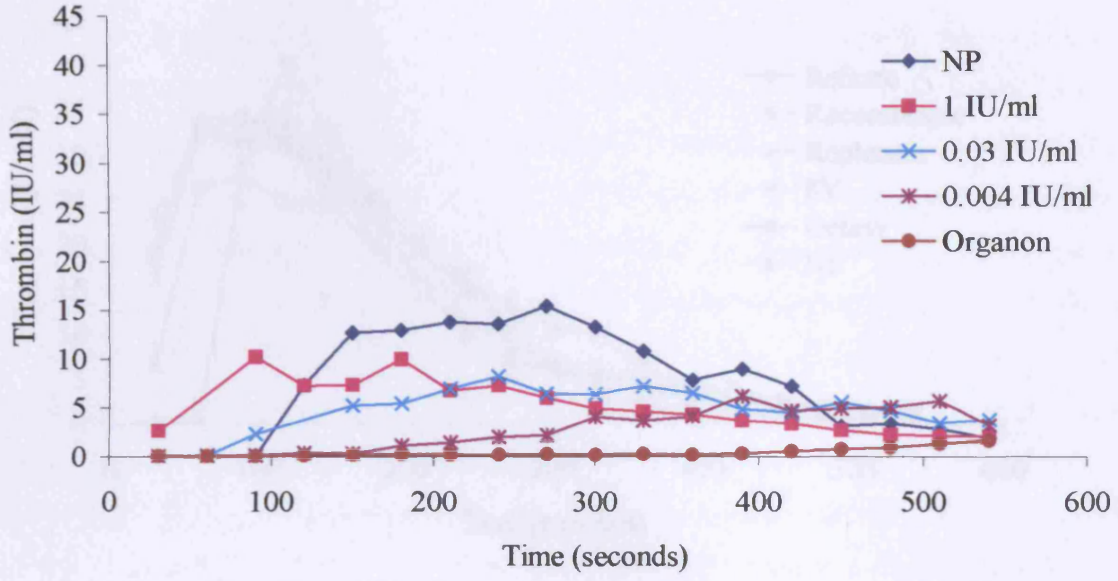
Fig 3.7 $T_{½max}$ values of thrombin generation for FVIII concentrates at a concentration of 0.03 IU/ml.

All experiments above were performed on defibrinated plasma. To see if there were any differences between the concentrates in the presence of fibrinogen or fibrin, thrombin generation was performed on un-defibrinated plasmas. Thrombin generation was carried out as before, the clot formed during the course of the assay, was wound onto a wooden stick and the plasma removed by squeezing the clot against the side of the tube. The clot itself was then left within the reaction mixture.

A dose-response was performed initially with *Replenate* with a PL concentration of 10μg/ml (Fig 3.8A). However, it was difficult to distinguish differences between FVIII concentrations. In addition, a greatly reduced amount of thrombin was generated in un-defibrinated plasma as compared to 3.3 above (these findings are explored in more detail in chapter 5). To enhance the differences between FVIII concentrations the PL concentration was increased to 50μg/ml resulting in a clearer dose-response (Fig 3.8B), and more thrombin generation.

A small group of FVIII concentrates were compared in un-defibrinated plasma (Fig 3.9) with a PL at 50μg/ml and FVIII concentrations of 1 and 0.03 IU/ml. No differences were observed in AUC, $T_{½max}$, and peak in un-defibrinated plasma.

A



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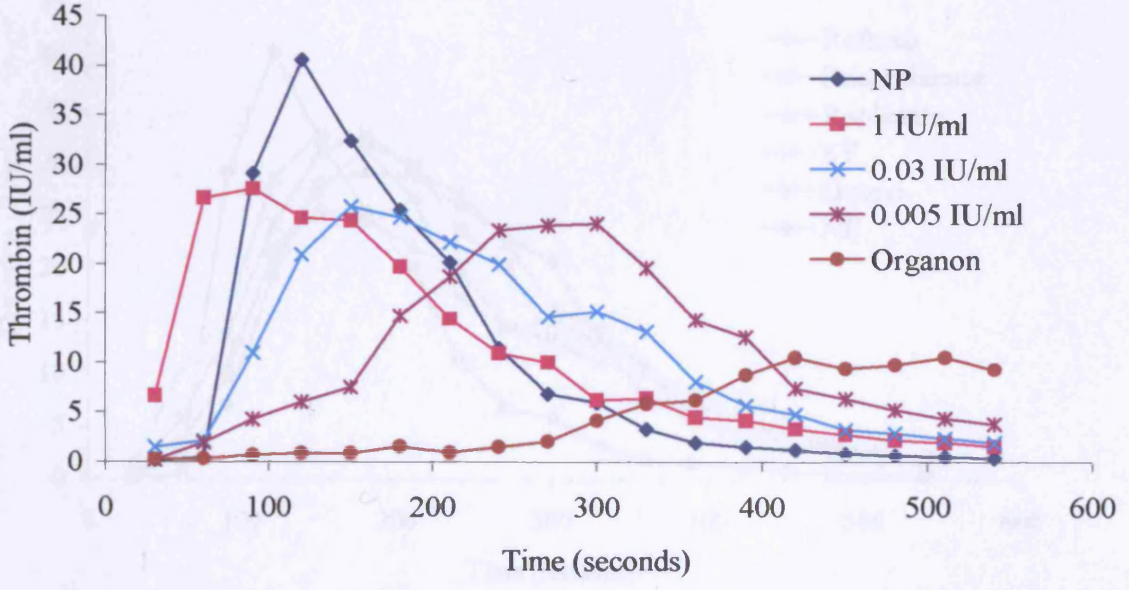
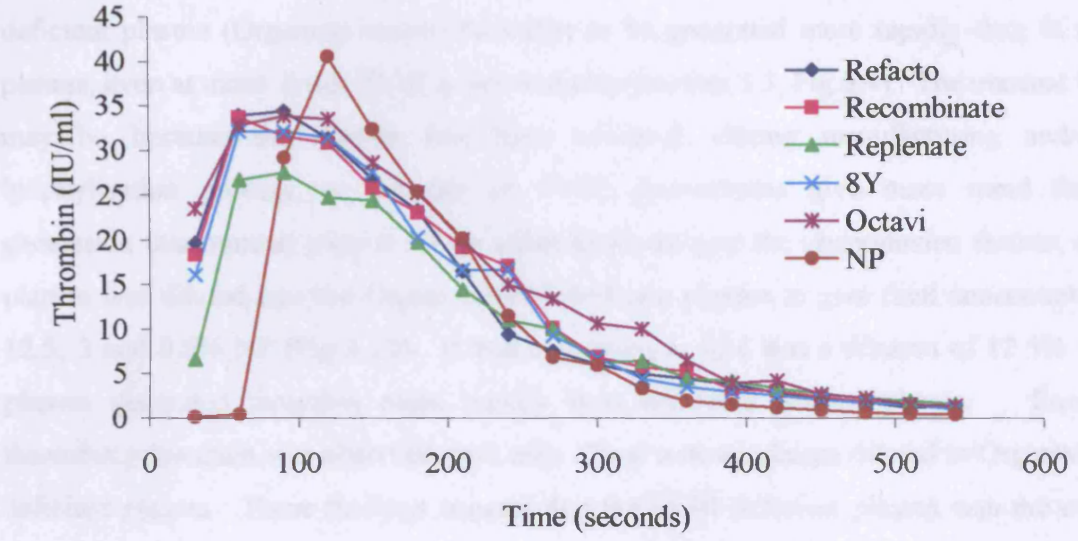


Fig 3.8 Dose-response of thrombin generation to FVIII in un-defibrinated plasma with two concentrations of PL. A) PL 10μg/ml, B) PL 50μg/ml. Replenate in Organon FVIII deficient plasma, n=2

A



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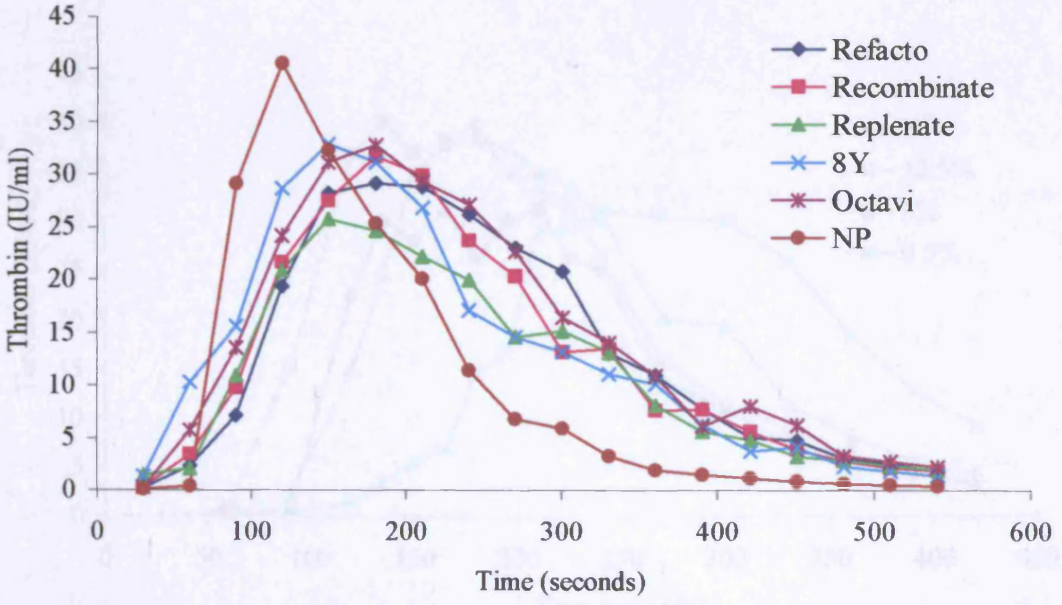


Fig 3.9 Thrombin generation by FVIII concentrates in un-defibrinated plasma at two FVIII concentrations.. A-1 IU/ml B 0.03 IU/ml, n=1

3.6 Investigation of rapid thrombin generation in Organon FVIII deficient plasma

It was noticed that adding FVIII concentrates to the commercial chemically depleted FVIII deficient plasma (Organon) caused thrombin to be generated more rapidly than in normal plasma, even at much lower FVIII concentrations (Section 3.3, Fig 3.4). The unusual finding may be because the plasma has been activated, during manufacturing and/or the lyophylisation process, or because all FVIII concentrates give more rapid thrombin generation than normal plasma. In an effort to investigate the phenomenon further, normal plasma was diluted into the Organon FVIII deficient plasma to give final concentrations of 12.5, 3 and 0.5% NP (Fig 3.10). It was surprising to find that a dilution of 12.5% normal plasma generated thrombin more rapidly than undiluted normal plasma. Equivalent thrombin generation was observed with only 3% of normal plasma diluted in Organon FVIII deficient plasma. These findings suggest that the FVIII deficient plasma was the cause of rapid thrombin generation rather than the concentrates, enhancing thrombin generation at all FVIII levels, but having a profound effect on thrombin generation at low FVIII levels.

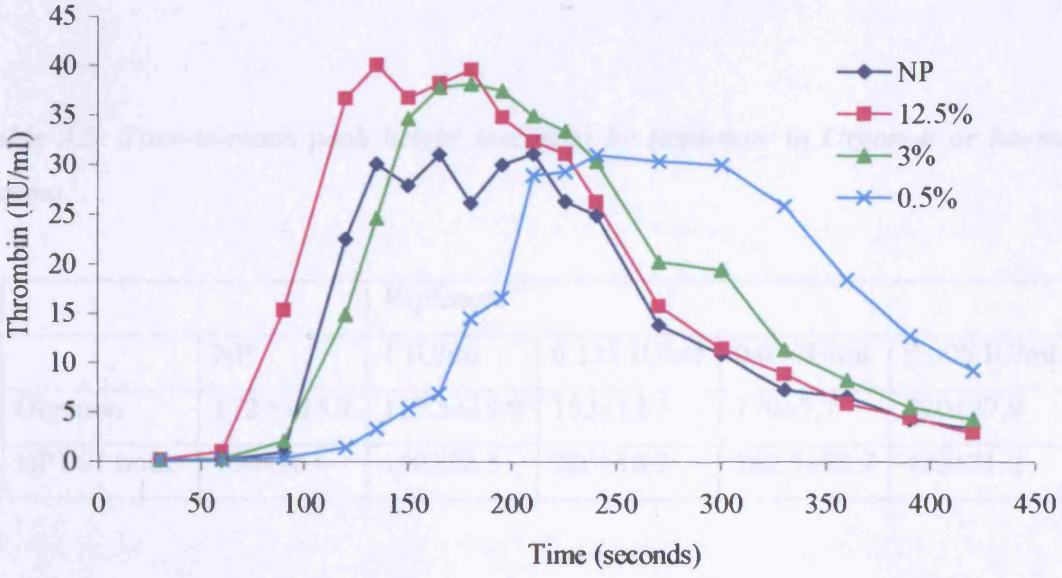


Fig 3.10 Thrombin generation by normal plasma in Organon FVIII deficient plasma. Normal plasma was diluted in Organon to give 12.5, 3 and 0.5% normal plasma..

To further ascertain if these findings were due to the choice of FVIII deficient plasma a dose-response was carried out by diluting *Replenate* in severe haemophilic plasma obtained from a patient 91 hours post-infusion and compared to Organon FVIII deficient plasma (Fig 3.11 Table 3.5). *Replenate* in Organon plasma consistently generated thrombin more rapidly than the same concentration of *Replenate* in haemophilic plasma, as demonstrated by shorter time-to-peak. These differences were significant between the time to reach peak-height at each FVIII concentration tested, $p<0.001$. Furthermore, in the Organon plasma the same time to peak-height as in normal plasma, occurred at 0.03 IU/ml whereas in haemophilic plasma this occurred at 1 IU/ml.

The reduced lag-time of thrombin generation which was observed in Organon (Fig 3.10) was not observed when normal plasma was diluted in haemophilic plasma (data not shown), or when *Replenate* was used in a different commercial deficient plasma which was prepared by monoclonal antibody depletion (Section 5.3.1, Fig 5.1).

Table 3.5 Time-to-reach peak height (seconds) by Replenate in Organon or haemophilic plasma.

		Replenate			
	NP	1 IU/ml	0.125 IU/ml	0.03 IU/ml	0.005 IU/ml
Organon	172.5±15.7	117.5±19.9	153±12.5	170±7.7	220±27.9
HP1 91 hours	180±10.6	190±22.5	220±18.2	262.5±22.7	420±21.2

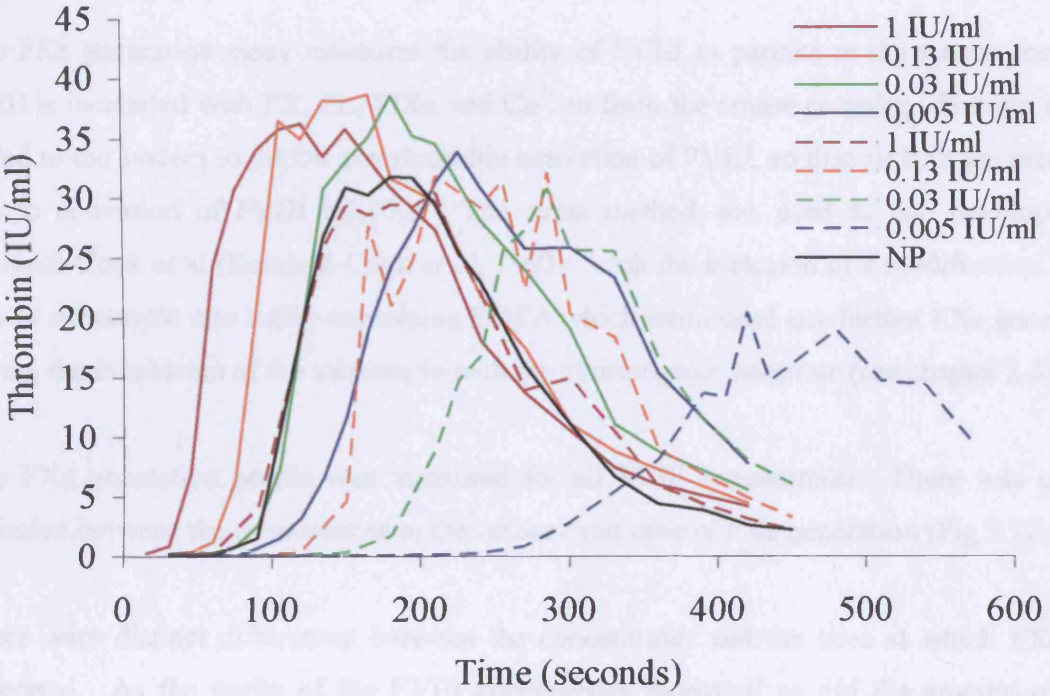


Fig 3.11. Effect on thrombin generation of different FVIII deficient plasmas over a range of FVIII concentrations. Bold lines-Replenate in Organon FVIII deficient plasma. Dashed lines-Replenate in severe haemophilic plasma.

These results suggest that the choice of FVIII deficient plasma is important in the TGT. The anomalies in lag-time with Organon plasma may be due to the way in which it was manufactured as this promotion of thrombin generation was not observed in haemophilic or in a commercial plasma prepared in a different manner.

3.7 Measurement of FXa generation

Thrombin generation curves were remarkably similar for all concentrates tested. From previous studies it is known that there are major differences in rate and amount of FXa generated by different FVIII concentrates (Kemball-Cook *et al*, 1993). The FXa assay was used to investigate differences between the FVIII concentrates used in this study and to examine why these differences occur.

The FXa generation assay measures the ability of FVIII to partake in the tenase complex. FVIII is incubated with FX, PL, FIXa and Ca^{2+} to form the tenase complex. Hirudin is also added to the system to inhibit any thrombin activation of FVIII, so that all FXa generation is due to activation of FVIII by FXa. The same method was used as that developed by Kemball-Cook et al (Kemball-Cook *et al*, 1993) , with the inclusion of a modification. This was to subsample into buffer containing EDTA which terminated any further FXa generation during the incubation of the subsample with the chromogenic substrate (see chapter 2.3)

The FXa generation profile was measured for all FVIII concentrates. There was a wide variation between the concentrates in the amount and time of FXa generation (Fig 3.12-3.14).

There were distinct differences between the concentrates and the time at which FXa was generated. As the purity of the FVIII concentrates increased so did the amount of FXa generated. Recombinant FVIII products all produced FXa at around the same time following a lag-time of 30 seconds, followed by the high purity concentrates, in which there was considerable difference between the ion-exchange and monoclonal antibody purified products. The intermediate purity *8Y* produced less FXa and later than all the other concentrates (Fig 3.12), this was similar to previous findings.

Refacto generated FXa most rapidly ($T_{½max}$ 61±4.3 seconds) followed by *Kogenate* (71±2.52 seconds) then *Recombinate* (79.2±6.3) these were significantly different from *Refacto* $p<0.05$, 0.001 respectively. The difference in $T_{½max}$ was also found to be significant between the two full-length recombinant products $p<0.05$. There were significant differences between all the other concentrates in the $T_{½max}$ (Fig 3.13) $p<0.001$.

Despite these differences in the time at which FXa was generated amongst the recombinant products, this was not translated into differences in the rate at which FXa was generated (calculated from the linear portion of the curve) (Fig 3.13). There was no difference between the recombinant products *Refacto* 11.5±1.3, *Recombinate* 11.6±2.1 and *Kogenate* 11.7±1.4 nM/min. There were however, significant differences between all the other concentrates in

the rate at which FXa was generated, *Replenate* 3.4±0.2, *Hemofil-M* 2.6±0.2, *Octavi* 0.75±0.06 and *8Y* 0.4±0.02 nM/min, P<0.001.

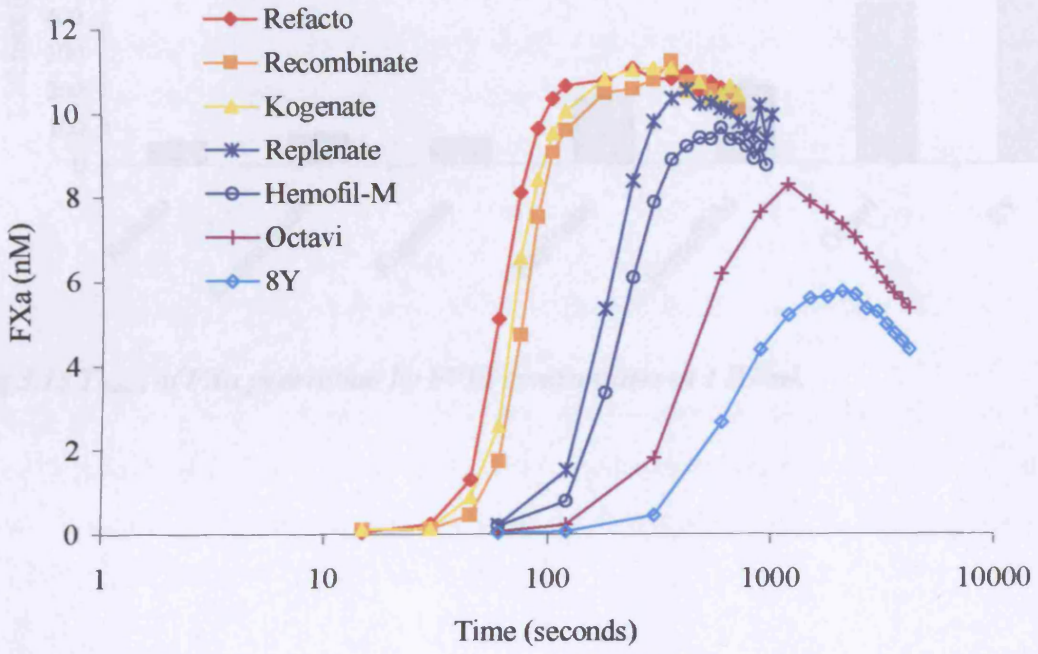


Fig 3.12 FXa generation by FVIII concentrates at a concentration of 1 IU/ml FVIII.

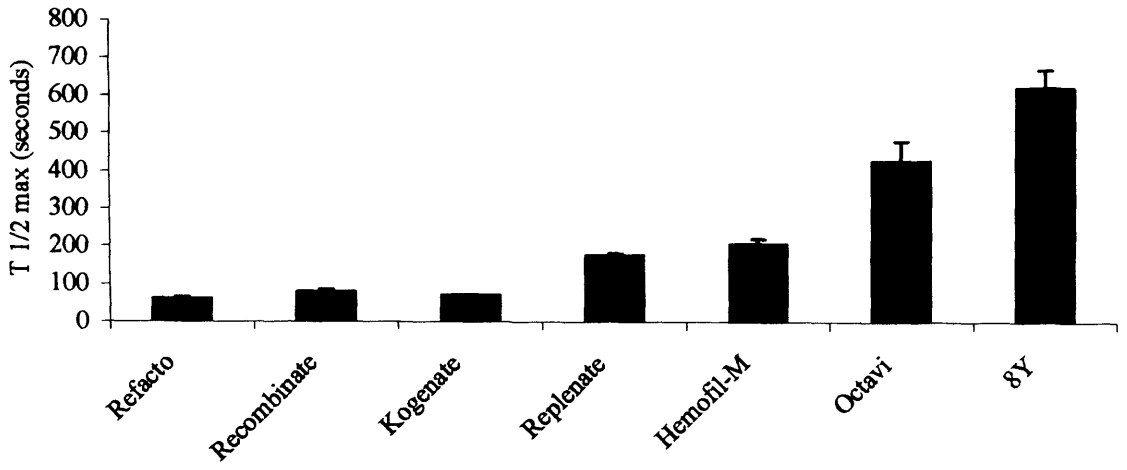


Fig 3.13 $T_{½max}$ of FXa generation by FVIII concentrates at 1 IU/ml.

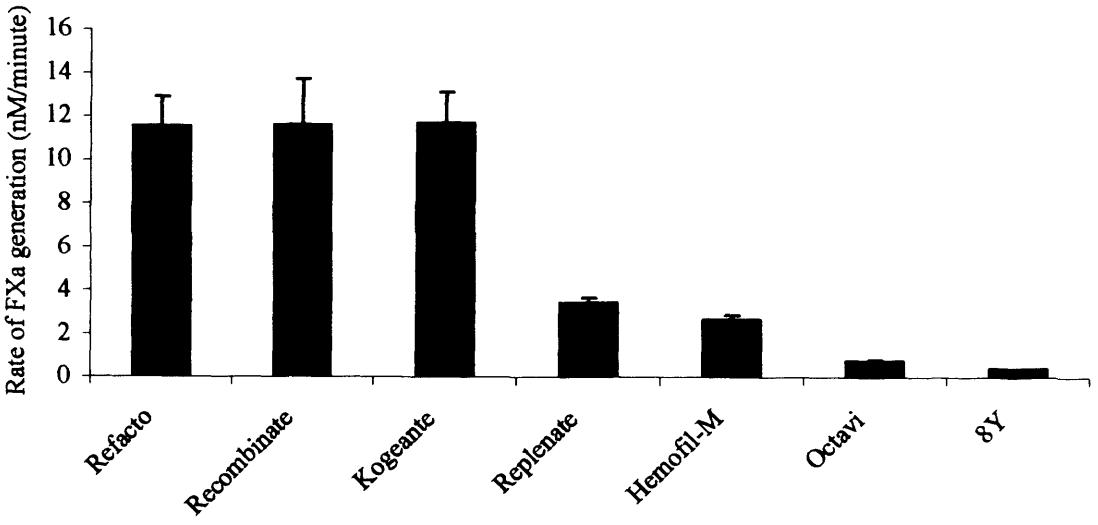


Fig 3.14 Rate of FXa generation by FVIII concentrates at 1 IU/ml

3.7.1 Thrombin activation

The next step was to investigate the effect of thrombin activation on FVIII and the effect this has on FXa generation, as FVIII *in vivo* is activated predominantly by thrombin and not FXa.

For these experiments two FVIII products were chosen; one full-length recombinant (*Kogenate*) and on intermediate purity concentrate (*8Y*). These two were chosen as *Kogenate* generated FXa rapidly when unactivated, whereas *8Y* was the slowest product to generate FXa.

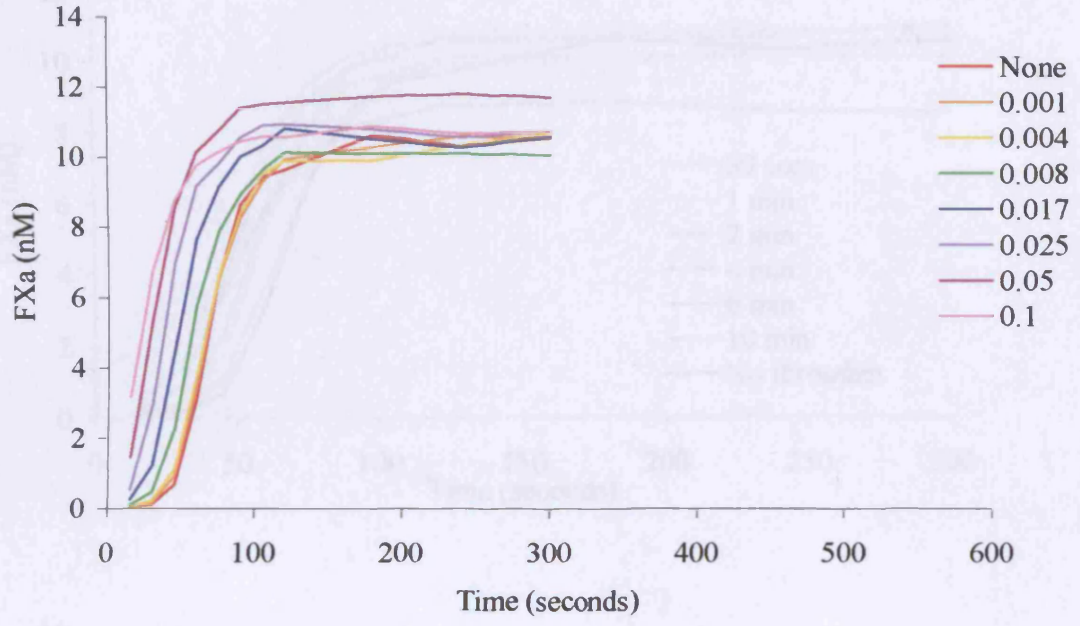
In the first instance FVIII activation was initiated with increasing concentrations of thrombin (0.001- 0.1IU/ml). Thrombin activation was then terminated after 30 seconds by addition of hirudin to the reaction mixture. Both concentrates reached maximal activation with 0.1 IU/ml thrombin (Fig 3.15). The results were more interesting however, at lower thrombin concentrations. Whereas *8Y* was sensitive to levels of 0.001 IU/ml, *Kogenate* lacked sensitivity until levels of 0.004 IU/ml were used.

The effect of incubation time (30seconds-10minutes) was then studied using a concentration of 0.004 IU/ml thrombin (Fig 3.16). Maximal thrombin activation occurred at 4 minutes for *Kogenate* and 6 minutes for *8Y*. Again *8Y* was more sensitive than *Kogenate* to shorter incubation times with just 15 seconds reducing the lag-time for FXa generation.

The final stage of these experiments was to study all concentrates with 0.004 and 0.1 IU/ml thrombin activation, to see if this reduced the differences between the concentrates in $T_{½max}$ and rate of FXa generation (Fig 3.17-3.19, Table 3.6, Table 3.7). A concentration of 0.004 IU/ml did not overcome the differences in FXa generation as there were still differences in both time and rate of FXa generation between the different types of concentrates, although the differences were reduced in comparison to no thrombin (Fig 3.12). The recombinant products reacted differently to the PD concentrates, in that at maximal thrombin activation although a significant reduction was observed in $T_{½max}$ $p<0.001$, there were no differences in the rate of FXa generation. The PD concentrates all produced a significant increase in FXa generation and a significant reduction in $T_{½max}$ values for both 0.004 and 0.1 IU/ml thrombin

activation, P<0.001. However even at maximal thrombin activation despite the reduction of the differences between the concentrates there was still variation between the concentrates in $T_{½max}$ and in the rate of FXa generation (ANOVA p<0.001 and <0.01 respectively). The shortest time to $T_{½max}$ was observed for the plasma derived concentrates *Hemofil-M* and *Replenate*, whilst the greatest amount of FXa generation was observed for the recombinant *Refacto* and the PD *8Y* and *Octavi*.

A



B

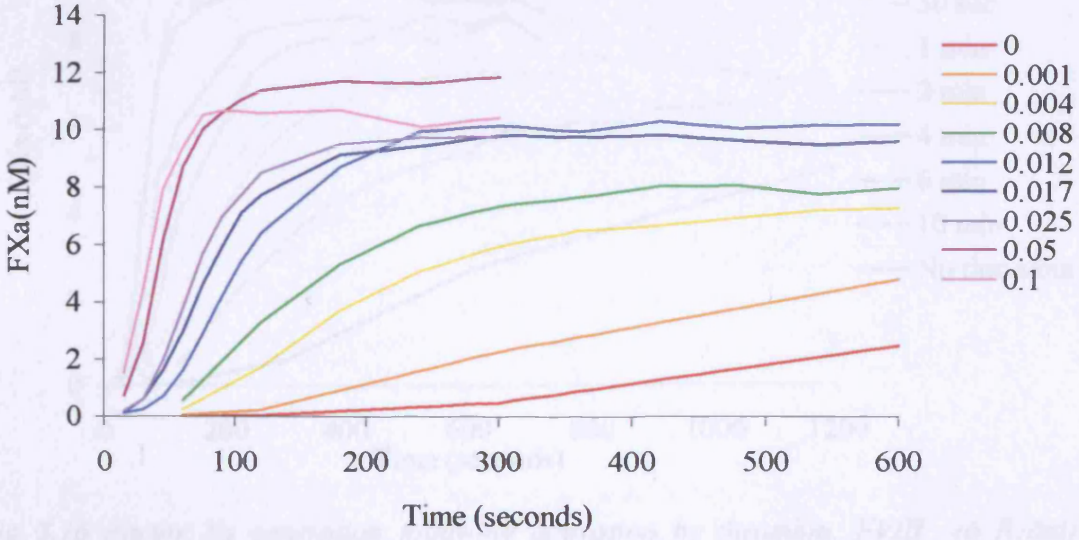
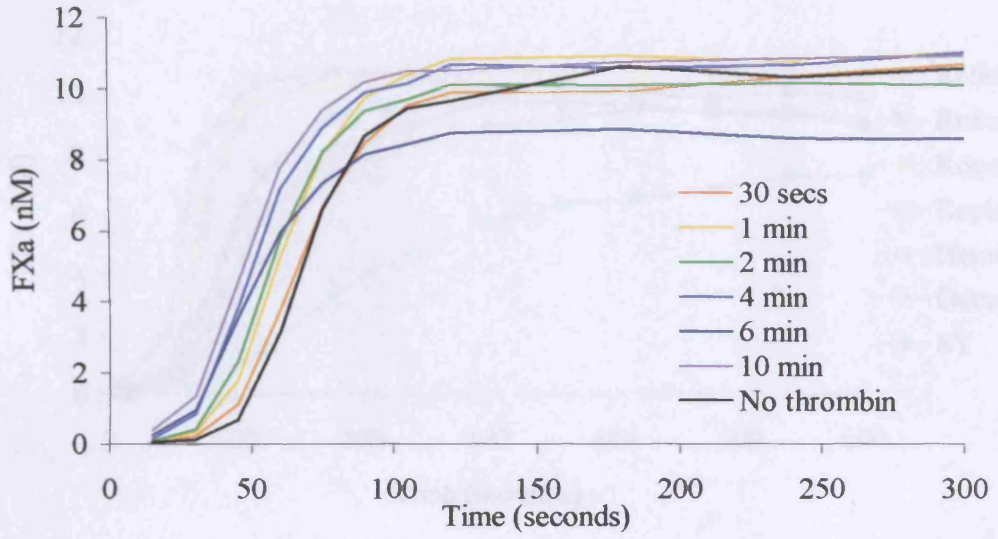


Fig 3.15 Effect of thrombin concentration on FVIII activation. FVIII (6 IU/ml) was activated by thrombin (0-0.1 IU/ml) for 30 seconds at 37°C. The activated FVIII was then added to hirudin, then to remaining reagents. A- Thrombin activation of Kogenate B- Thrombin activation of 8Y.

A



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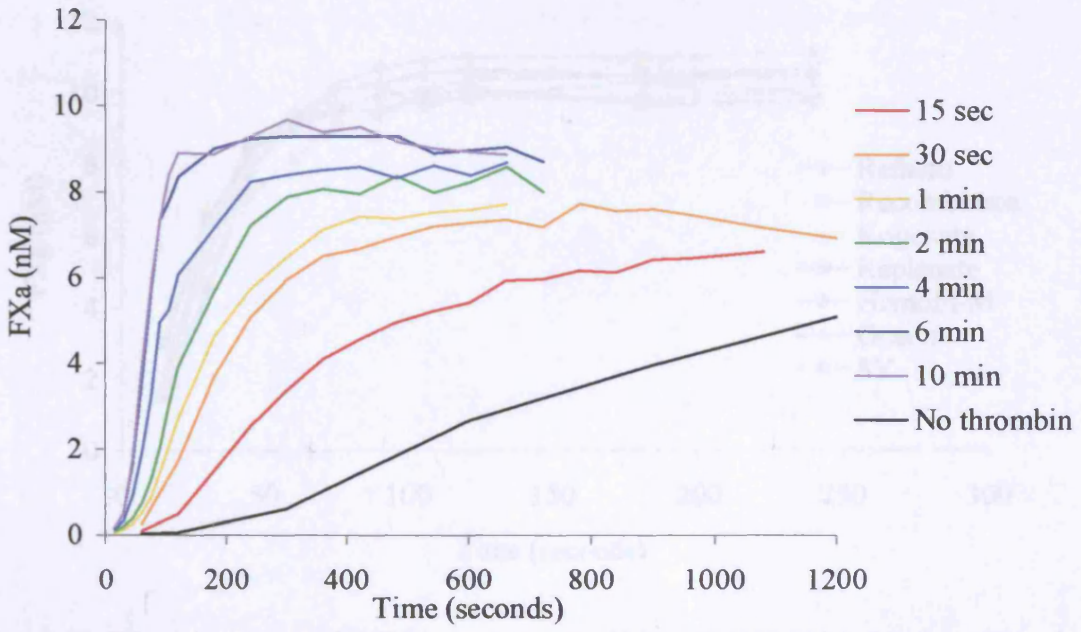
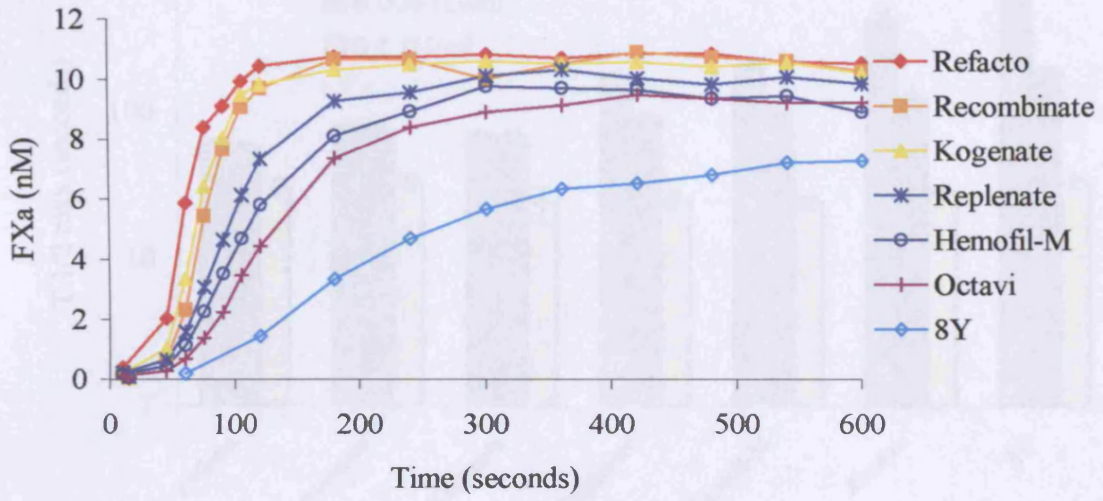


Fig 3.16 Factor Xa generation following activation by thrombin. FVIII (6 IU/ml) was activated by thrombin (0.004 IU/ml) for between 15 seconds and 10 minutes, FVIII was then added to hirudin, then remaining reagents. A- Thrombin activation of Kogenate, B- Thrombin activation of 8Y.

A



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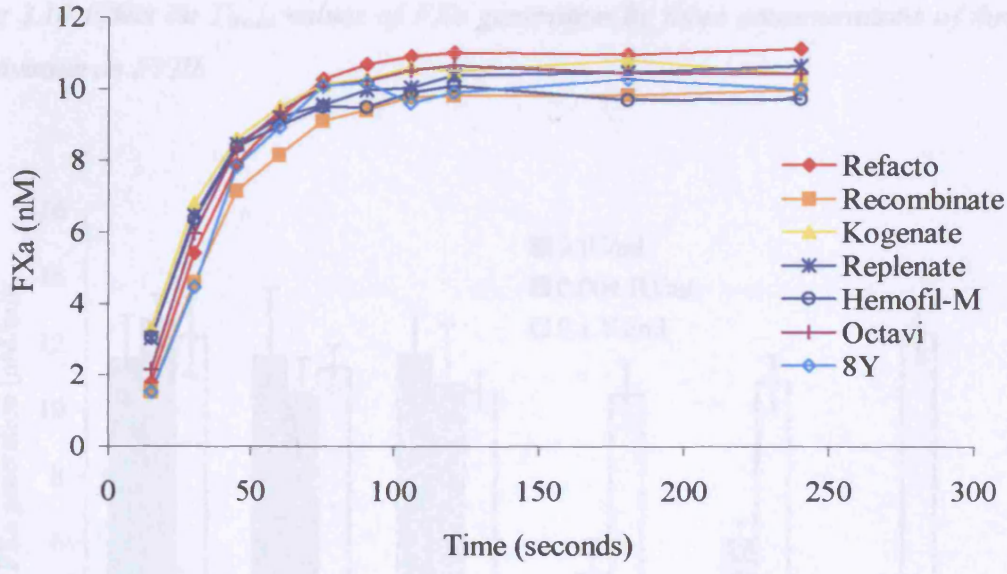


Fig 3.17 FXa generation of FVIII concentrates following activation by thrombin. A- FXa generation following activation by 0.004 IU/ml thrombin for 30 seconds. B- FXa generation following activation by 0.1 IU/ml thrombin for 30 seconds.

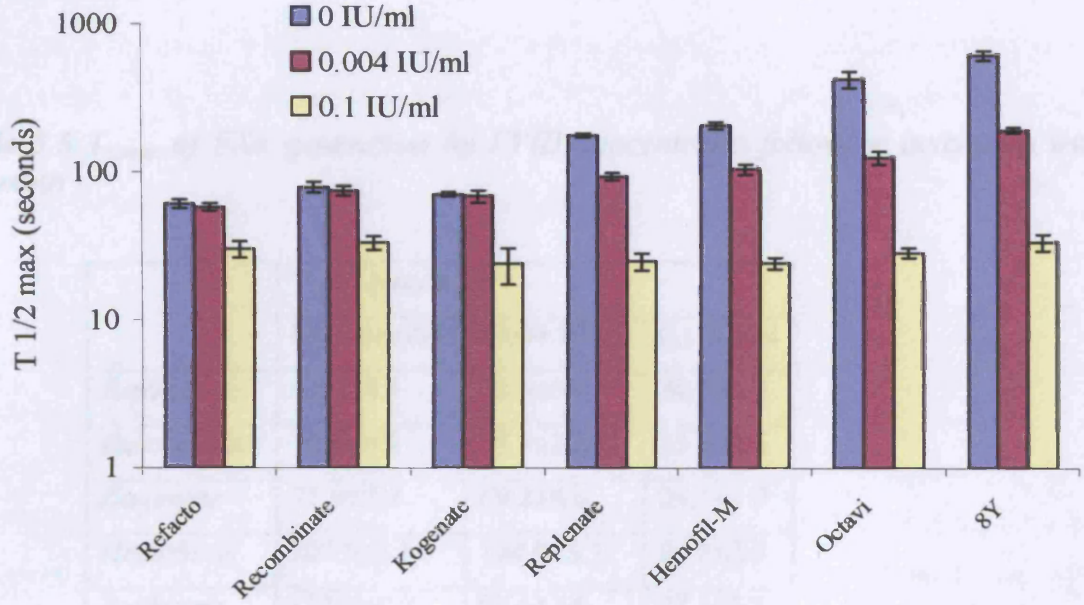


Fig 3.18 Effect on $T_{½max}$ values of FXa generation by three concentrations of thrombin on activation on FVIII.

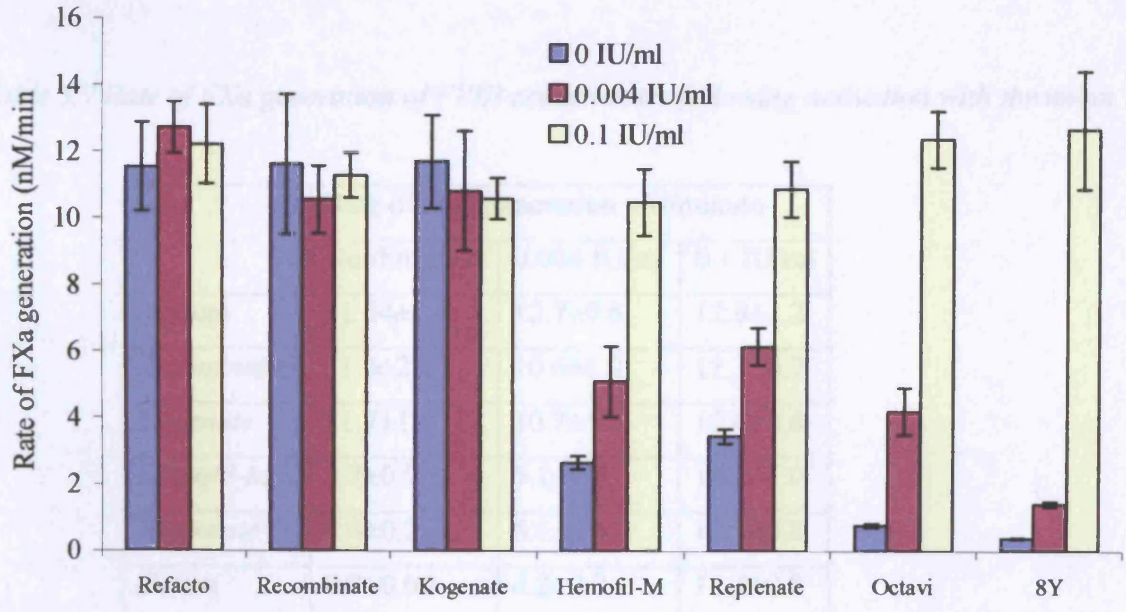


Fig 3.19, Rate of FXa generation following thrombin activation

Table 3.6 $T_{½max}$ of FXa generation by FVIII concentrates following activation with thrombin

	$T_{½max}$ (seconds)		
	No thrombin	0.004 IU/ml	0.1 IU/ml
Refacto	61.4±4.3	58.6±3.4	30.3±3.9
Recombinate	79.3±6.2	75.1±5.8	33.4±3.6
Kogenate	71.0±2.5	69.2±6.6	24.1±1.9
Hemofil-M	207.8±12.0	164.9±8.7	24.3±2.3
Replenate	177.4±5.2	93.6±5.6	25.1±3.3
Octavi	426.9±54.3	125.8±12.6	28.4±2.2
8Y	622.9±47.8	192.8±7.9	33.8±4.3

Table 3.7 Rate of FXa generation of FVIII concentrates following activation with thrombin

	Rate of FXa generation nM/minute		
	No thrombin	0.004 IU/ml	0.1 IU/ml
Refacto	11.54±1.4	12.7±0.8	12.8±1.2
Recombinate	11.6±2.1	10.6±1.0	11.3±0.7
Kogenate	11.7±1.4	10.7±1.8	10.6±0.6
Hemofil-M	2.7±0.2	5.1±1.1	10.5±1.0
Replenate	3.4±0.2	6.1±0.6	10.9±0.8
Octavi	0.8±0.06	4.2±0.7	12.4±0.8
8Y	0.4±0.02	1.4±0.07	12.6±1.8

3.7.2 Effect of VWF

It is thought that the amount of VWF in FVIII concentrates may affect the amount and rate of FXa generation, as VWF may interfere with FVIII activation. This may explain why in earlier experiments *8Y* which contains VWF is always slower and produces less FXa compared with more highly purified FVIII products.

In this experiment, PD VWF (kind gift of Prof Mazurier, LFB, Lille, France) which contained VWF:Ag 200 IU/ml was added to the recombinant concentrate *Kogenate*. For this experiment *Kogenate*, which was from a product vial, reconstituted and snap frozen in aliquots, was used as opposed to the ampuoled NIBSC material. This change in *Kogenate* was necessary, as a higher concentration was needed in order to test a number of VWF concentrations. VWF was added to FVIII for 5 minutes at 37°C before addition to remaining FXa reagents (PL, FX, FIXa, hirudin, Ca^{2+}), and the assay was carried out as previously described. The results showed (Fig 3.20) a prolongation in the time for FXa to be generated and a decrease in the amount of FXa formed when increasing VWF was present. For the curves of *8Y* and *Kogenate* to have a similar lag-time for FXa generation, VWF was required at a ratio of 10:1, this is far in excess of the amount of VWF found in *8Y* which is present at a ratio of 3:1 (Raut *et al*, 1999).

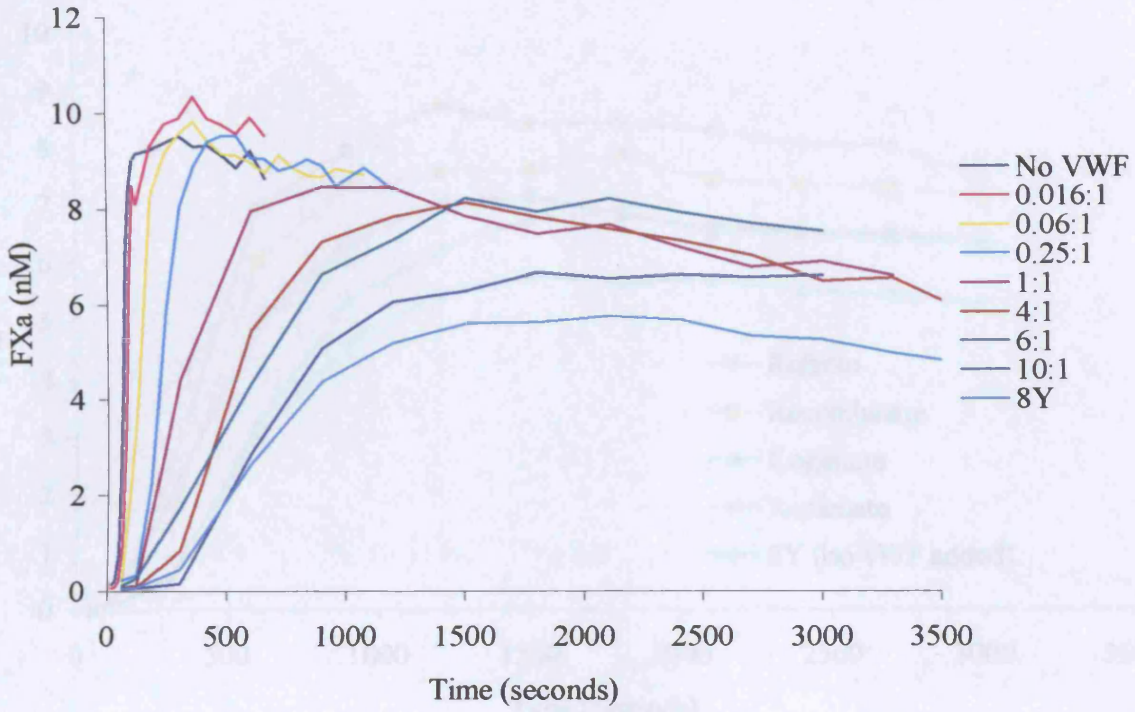


Fig 3.20 Effect of VWF on FXa generation by Kogenate, ratio of VWF:Ag toFVIII:c. 8Y was included for comparison (3:1), n=1.

Subsequent experiments were performed with a range of FVIII concentrates of differing purity and adding VWF to these concentrates at a ratio of 4:1 (Fig 3.21). It was found that in these experiments a ratio of 4:1 VWF when added to *Kogenate* caused equivalent FXa generation compared to *8Y*, there was no significant differences between the two concentrates in $T_{½max}$ (Fig 3.22) or in rate of FXa generation (Fig 3.23). However, the other concentrates (*Refacto*, *Recombinate*, and *Replenate*) generated FXa more rapidly, with shorter $T_{½max}$ values, this was found to be significantly different from *8Y* and *Kogenate* $p<0.01$. These concentrates also generated FXa more rapidly with *Refacto* and *Recombinate* generating FXa at a rate twice that of *8Y* and *Kogenate*. *Refacto*, *Recombinate* and *Replenate* were also found to generate FXa at a significantly faster rate than *8Y* and *Kogenate* $p<0.01$.

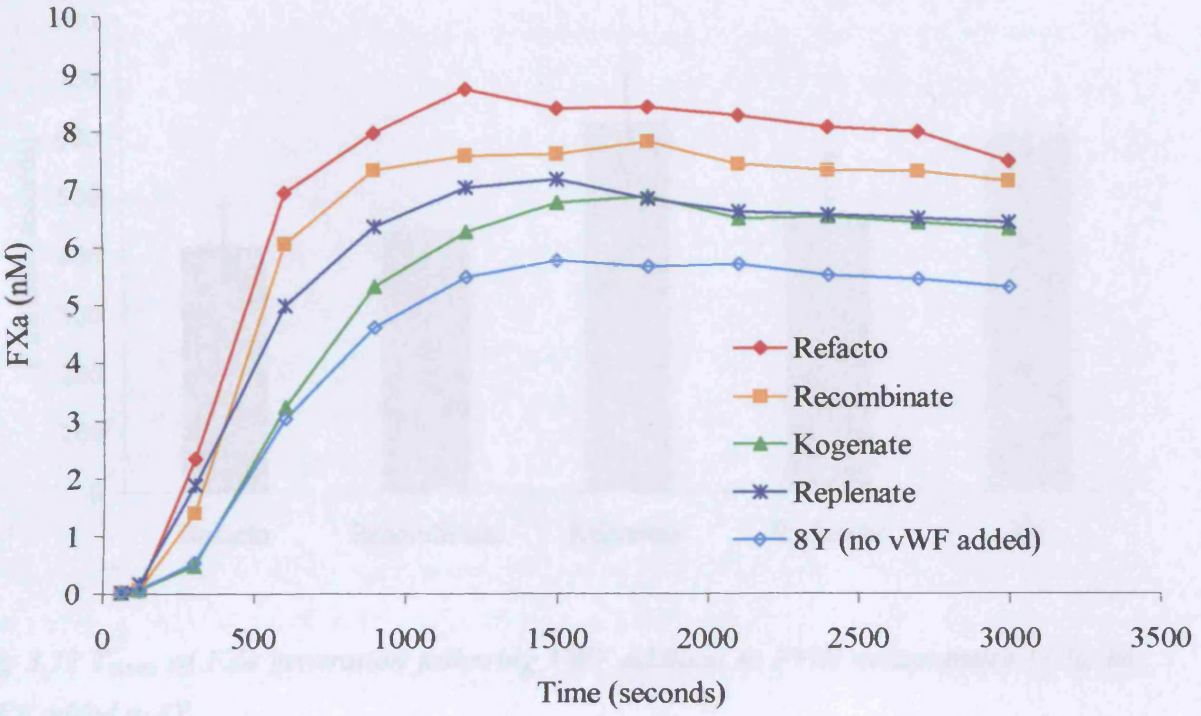


Fig 3.21. FXa generation of FVIII concentrates with the addition of VWF to a ratio of 4:1, no additional VWF added to 8Y.

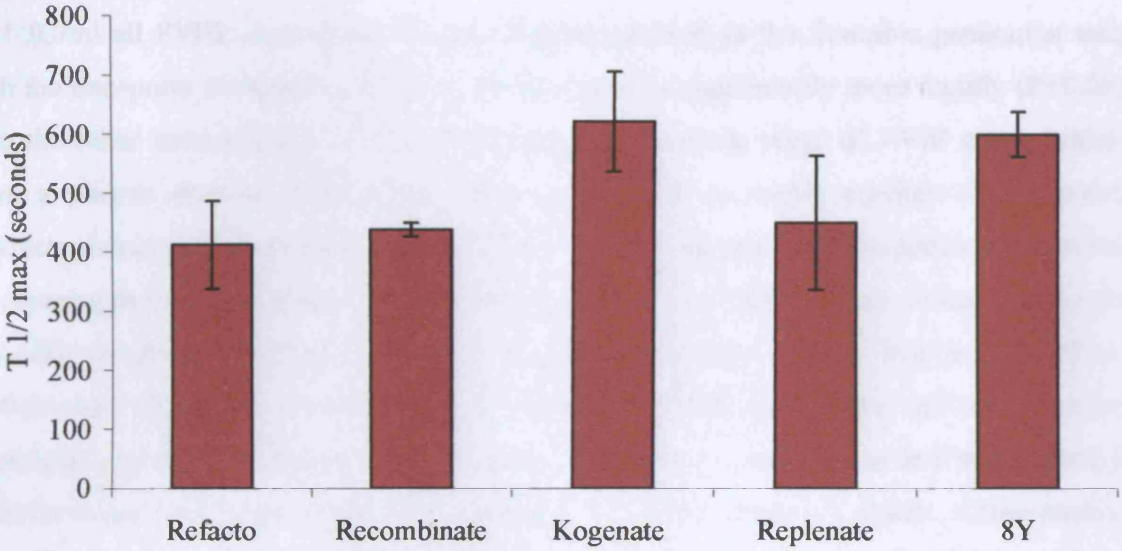


Fig 3.22 $T_{½max}$ of FXa generation following VWF addition to FVIII concentrates (4:1), no VWF added to 8Y.

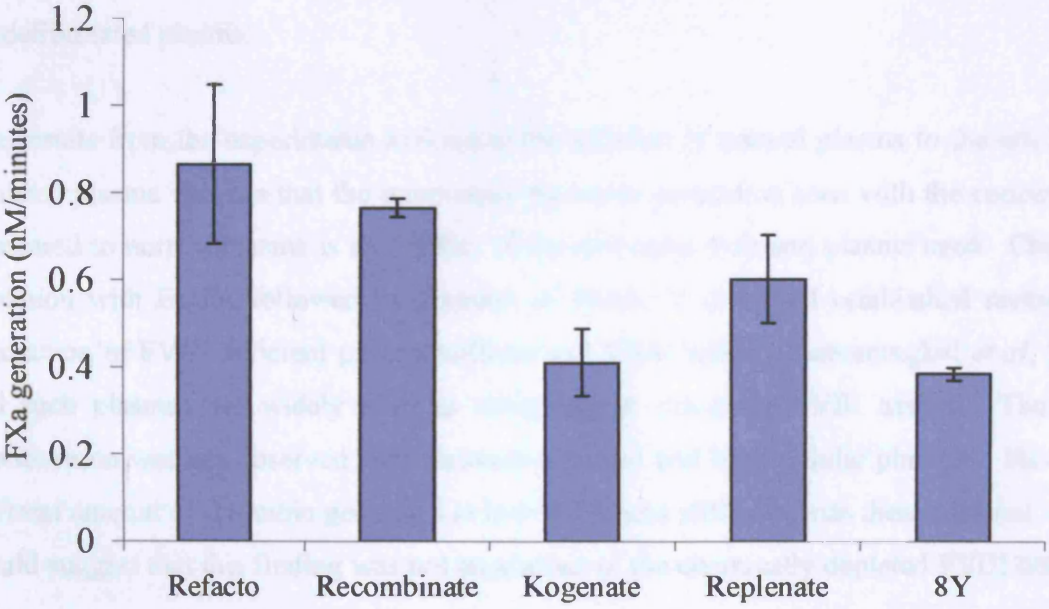


Fig 3.23 Rate of FXa generation following addition of VWF to FVIII concentrates (4:1) except 8Y.

3.8 Discussion

At 1 IU/ml all FVIII concentrates tested behaved similarly in the thrombin generation test, with the exception of *Refacto* which produced thrombin significantly more rapidly ($P<0.05$) than the other concentrates. It was interesting that the wide range of FVIII concentrates, from a plasma derived intermediate purity concentrate to highly purified recombinants products should produce thrombin at the same rate. This suggests that the potencies obtained by chromogenic assays using high dilutions give a good prediction of their ability to generate thrombin at physiological concentrations, and hence possibly of their haemostatic effect. *Refacto* was the only concentrate to be significantly different in the rate of thrombin generation at 1 IU/ml. *Refacto* is a high purity recombinant concentrate and is structurally different from other recombinant concentrates in that the B domain is absent. Other studies have found BDD FVIII to be functionally comparable to full length FVIII products (Sandberg *et al*, 2001) except in the ability to bind to phospholipids. This difference however, is reduced with the use of a physiological source of phospholipid such as platelets (Mikaelsson *et al*, 1998). Despite the differences observed between concentrates at 1 IU/ml, no other substantial differences were observed at a lower concentration of 0.03 IU/ml or in un-defibrinated plasma.

The results from the experiments looking at the addition of normal plasma to the artificially depleted plasma indicate that the more rapid thrombin generation seen with the concentrates compared to normal plasma is an artefact of the particular deficient plasma used. Chemical depletion with EDTA followed by addition of Factor V is a well established method for production of FVIII deficient plasma with normal VWF levels (Chantarangkul *et al*, 1978), and such plasmas are widely used as substrates in one-stage FVIII assays. The same phenomenon was not observed with immuno-depleted and haemophilic plasmas. However, the total amount of thrombin generated at low levels was still high with these plasmas, which would suggest that this finding was not an artefact of the chemically depleted FVIII deficient plasma.

There were large differences between the concentrates in the ability to generate FXa in a purified system. FXa was generated most rapidly by the recombinant products, and slowest

by the intermediate purity product *8Y*. These results are in agreement with those found by Kemball-Cook et al in a similar FXa generating assay (Kemball-Cook *et al*, 1993), although they attributed the differences between the concentrates to the presence of FVIIIa. We have however, demonstrated that differences in the rate of FXa generation are due to the VWF content of the concentrates, as the addition of VWF to the concentrates caused a delay and decreased the amount of FXa generated. This effect of VWF has also been observed by Koppelman et al in which VWF was found to inhibit FXa generation, if present at the start of the reaction (Koppelman *et al*, 1994). These findings may be explained by the observation that VWF inhibits cleavage of FVIII by FXa (Koedam *et al*, 1990). Furthermore, they suggest that in a purified system, FVIII in complex with VWF is less able to support FXa generation following activation by FXa. It has also been suggested that VWF prevents FVIII from binding to PL prematurely (Saenko *et al*, 1999a) and this may also account for the increased lag-time when VWF is present, as without FVIII binding to PL the tenase complex is unable to form. This corresponds to our findings in which FXa generation was delayed and a smaller amount produced in those concentrates containing VWF. However, addition of an equal amount of VWF to a range of highly purified FVIII concentrates did not result in the same FXa generation profile as that of the intermediate purity product *8Y*, and would suggest that although the majority of the differences in FXa generation is due to VWF, this does not account for all variation.

Differences in generation of FXa were observed between the concentrates upon treatment with thrombin, with a concentrate containing VWF showing increased FXa generation with a low concentration of thrombin, whereas the same concentration of thrombin had no effect on a recombinant product. Other work has shown that thrombin cleavage of FVIII is enhanced by the presence of VWF which increases cleavage in the light chain of FVIII (Arg 1689) by eight-fold (Hill-Eubanks & Lollar, 1990). This would explain our findings in that at low concentrations of thrombin or with short incubation times concentrates containing high amounts of VWF show a dramatic response. This is most likely due to VWF increasing the cleavage of FVIII, so that a greater proportion of the FVIII present is in the active form before the termination of the thrombin activation step by hirudin, and is thus able to generate

FXa more rapidly. However, following activation with low concentrations of thrombin, FXa generation is still more rapid in concentrates which do not contain VWF.

Upon full thrombin activation of the FVIII concentrates there were still small differences in the time and rate of FXa generation. However, these differences do not seem to discernibly affect thrombin generation as the only difference observed was the decreased time for thrombin generation to occur for *Refacto*, even though FXa was not more rapid than any other recombinant concentrate when fully activated by thrombin. The differences between the FXa assay and the TGT and differences between concentrates are because they are measuring different things. The FXa assay is measuring one component in the pathway to thrombin generation. Only a small amount of FXa is required for full prothrombin activation. In addition, large amounts of thrombin are generated in the TGT which is much greater than the concentration required for full activity in the FXa generating assay, which will produce full and rapid activation of FVIII in the TGT. Also differences in VWF content are equalled out in the TGT by use of FVIII deficient plasma containing normal VWF levels.

3.9 Summary

Differences were observed between the concentrates in a FXa generating assay, with the most rapid FXa generation by recombinant products and the slowest FXa generation by an intermediate purity product. These differences were mainly due to the different VWF content of the concentrates and there was no difference in FXa generation when the concentrates were fully activated by thrombin. Despite the differences in FXa generation the only difference observed in thrombin generation was that *Refacto* generated thrombin more rapidly than the other concentrates at 1 IU/ml.

CHAPTER 4
STUDIES AT LOW FVIII LEVELS

4.1 Introduction

Findings from the previous chapter demonstrated a surprisingly large amount of thrombin generation even at FVIII concentrations of 0.005 IU/ml. This was an unanticipated finding as the sensitivity of the one-stage APTT and chromogenic assays are generally 0.01 IU/ml. A concentration of FVIII <0.01 IU/ml is associated with a severe clinical profile in which spontaneous bleeding occurs, thus a much reduced thrombin generation profile would be anticipated at these severe FVIII levels. The experiments in this chapter further investigate the sensitivity of the TGT to FVIII.

Another avenue that is explored in this chapter is the protection of FVIII against anti-FVIII antibodies by a complex of FIXa and PL. Previous work by Barrowcliffe et al had demonstrated protection of FVIII by FIXa and PL, although this protection was mostly due to the action of PL (Barrowcliffe *et al*, 1983). In light of our findings, i.e. a large amount of thrombin generated by small amounts of FVIII it was decided to re-investigate the role of FIXa in protecting FVIII from antibodies.

4.2 Results with haemophilic plasma

It was observed that some variation was occurring in the amount of thrombin generated by the commercial FVIII deficient plasma (Organon) between batches. Also, as the concentrates had been tested in a commercial plasma it was desirable to test the findings in severe haemophilic plasma. Commercial plasma is preserved by lyophilisation and may therefore be unphysiological.

4.2.1 Thrombin generation in two severe haemophilic plasmas

Initially thrombin generation was measured in two severe haemophilic patients. The plasma was obtained from HP1 at 37 hours post-infusion, the sample taken from HP2 was obtained at 72hours post-infusion. These plasmas generated a large amount of thrombin (Fig 4.1) with

AUC almost two thirds that of normal plasma (HP1 4532±753, HP2 4765±339, NP 6934±749 IU/ml.s). This finding was unexpected as both plasmas had been measured by one-stage APTT assay and found to contain less than 0.01 IU/ml FVIII. More plasma was obtained from HP1 at 61 and 91 hours post-infusion, these generated considerably less thrombin (AUC 331±253 IU/ml.s at 61 hours 1414.4±260.6 IU/ml.s at 91 hours post-infusion) than at the earlier time point. As all samples contained less than 0.01 IU/ml FVIII, this suggests that thrombin is generated by FVIII below this level. The reduction in thrombin generation from patient HP1 with plasma taken at a longer duration post-infusion suggests that more FVIII was present in the original sample, due to the presence of residual FVIII concentrate. This is in contrast to patient HP2 in which the original sample was taken at 72 hours post-infusion and suggests that thrombin generation in this patient may be due to a small amount of endogenous FVIII.

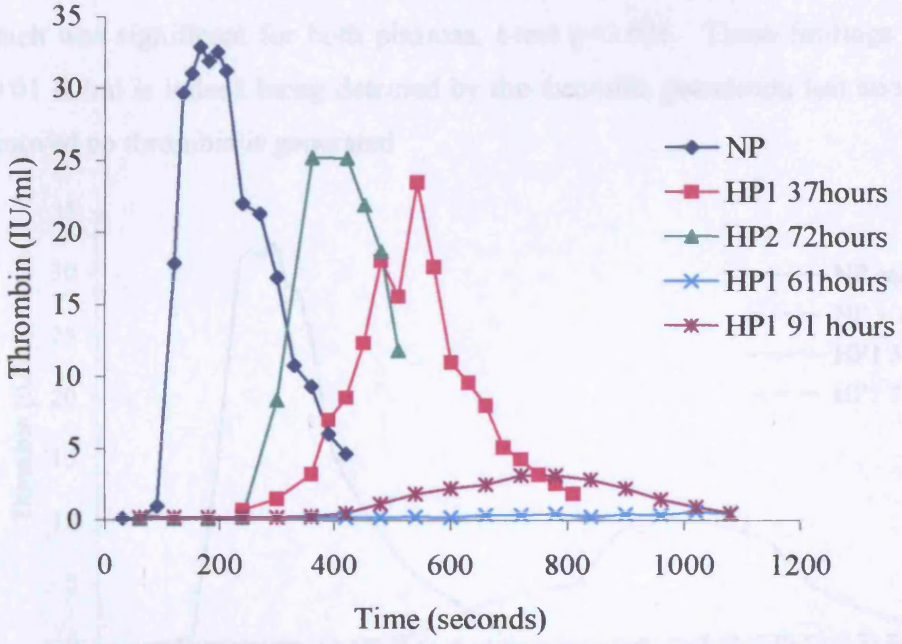


Fig 4.1. Thrombin generation by normal plasma and two haemophilic patients. Three samples were obtained from one patient (HP1) at 37, 61 and 91 hours post-infusion, and patient 2(HP2) at 72 hours.

To confirm that the large amount of thrombin generated by HP1 at 37 hours post-infusion was due to FVIII, the plasma was incubated with a polyclonal anti-FVIII antibody (Ab) overnight (Fig 4.2). If thrombin generation was not due to residual FVIII in the plasma then no difference in the amount of thrombin generated by control and anti-FVIII Ab would be observed.

To test this a rabbit polyclonal anti-FVIII Ab was added to normal and HP1 37hours at a dilution of 1 in 1000 for the FVIII Ab and at 10μg/ml for the control Ab (Gammabulin). The plasma and antibody mixtures were incubated at 37^0C overnight. The thrombin generation test was then carried out as before.

Treatment with anti-FVIII Ab abolished thrombin generation in both normal (AUC 5514.75±551.5 IU/ml.s for control to 329.3±110.0 IU/ml.s for anti-FVIII Ab) and the haemophilic plasma (AUC 3655.4±661.3 to 146.4±38 IU/ml.s for anti-FVIII Ab treated), which was significant for both plasmas, t-test $p<0.001$. These findings suggest that FVIII <0.01 IU/ml is indeed being detected by the thrombin generation test and that if all FVIII is removed no thrombin is generated

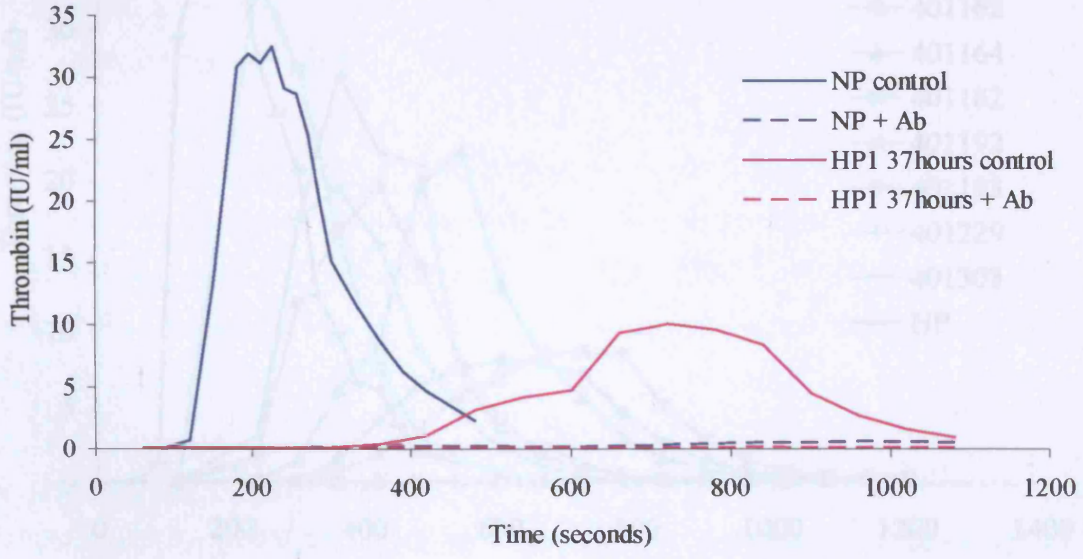


Fig 4.2. Thrombin generation of normal and haemophilic plasma following overnight incubation at 37 °C with polyclonal rabbit anti-FVIII antibody or control (gammabulin).

4.2.2 Prophylaxis study

Due to the high sensitivity of the thrombin generation test it was decided to use the TGT in a clinical setting by examining the thrombin generation profile of a group of severe haemophilic children on prophylaxis regimes. The children were 6-14 years old and all received prophylaxis treatment three times a week with 500 or 1000 U of *Refacto* or *Kogenate*.

Samples were obtained from 8 children that attended clinic during a five month period. Samples were measured for thrombin generation using the clotting method with a high FIXa concentration to start the reaction. The samples were collected at 54-128 hours post-infusion. The FVIII level of the samples was measured by one-stage assay on the ACL 10,000 at the Royal Free Hospital by Dr Dale Owens.

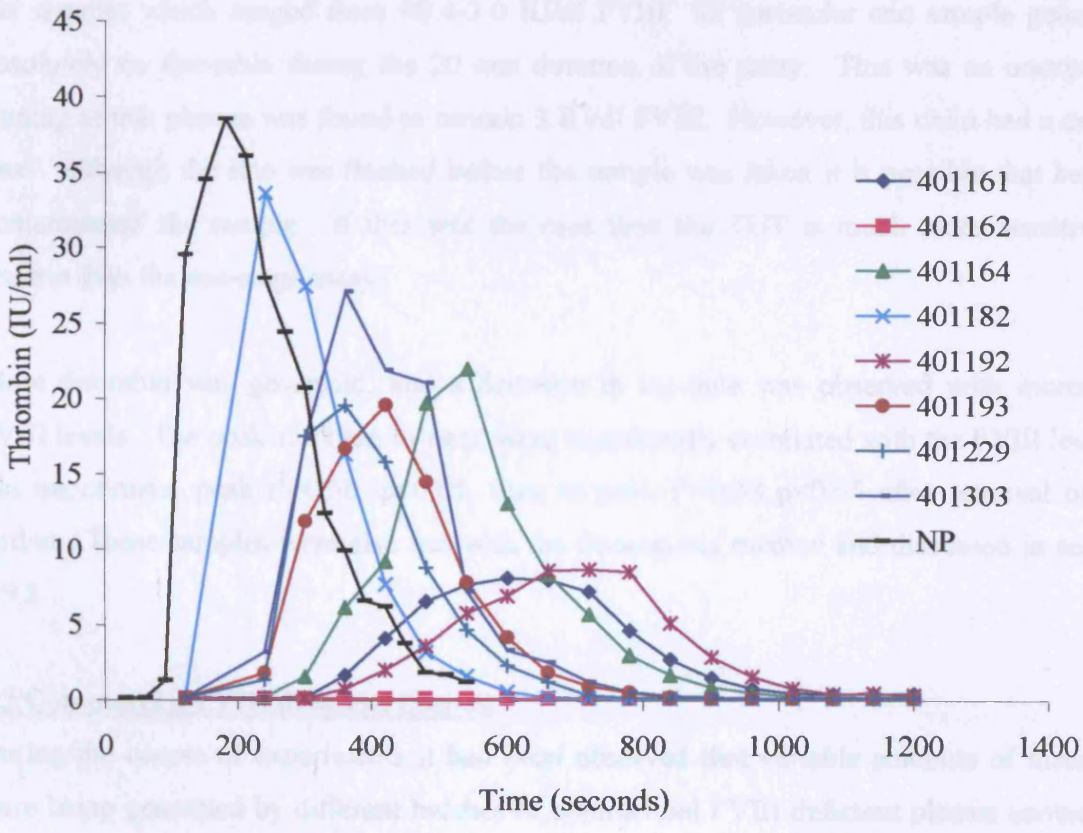


Fig 4.3 Thrombin generation by severe haemophilic patients at trough FVIII levels during prophylaxis treatment (n=1)

Table 4.1 Thrombin generation parameters of prophylaxis samples as a percentage of normal plasma (m=1)

Sample ID		401161	401162	401164	401182	401192	401193	401229	401303
FVIII IU/dl		0.4	3.0	0.6	2.7	0.4	0.6	1.0	0.8
% of NP	AUC	43.23	2.53	73.08	85.26	44.92	64.19	57.65	82.41
	Peak	21.86	-	59.54	83.74	22.10	48.85	58.12	67.52
	Time to peak	323.08	-	261.54	123.08	353.85	215.38	169.23	184.62

There was a wide variation in the amount of thrombin generated (Fig 4.3 Table 4.1) between the samples which ranged from <0.4-3.0 IU/dl FVIII. In particular one sample generated absolutely no thrombin during the 20 min duration of the assay. This was an unexpected finding as this plasma was found to contain 3 IU/dl FVIII. However, this child had a central line. Although the line was flushed before the sample was taken it is possible that heparin contaminated the sample. If this was the case then the TGT is much more sensitive to heparin than the one-stage assay.

More thrombin was generated, and a decrease in lag-time was observed with increasing FVIII levels. The peak and time-to-peak were significantly correlated with the FVIII level in this experiment, peak $r^2=0.58$, $p<0.05$, time to peak $r^2=0.58$ $p<0.05$ after removal of the outlier. These samples were also run with the fluorogenic method and discussed in section 5.9.2

4.3 Commercial FVIII deficient plasma

During the course of experiments it had been observed that variable amounts of thrombin were being generated by different batches of commercial FVIII deficient plasma containing less than 0.01 IU/ml FVIII. It was decided to investigate this more fully by investigating Organon and a range of commercial FVIII deficient plasmas.

The five most widely used commercial plasmas were selected from a UK NEQAS study (April 2001). These were: Dade, Technoclone, Sigma , IL and Organon. Organon is manufactured by a chemical process, the other plasmas are produced using an immuno-depletion method with antibodies against FVIII or VWF. Organon, Technoclone and Dade all contain normal levels of VWF, whereas the plasmas by Sigma and IL are also deficient in VWF. All plasmas were assayed for FVIII in a chromogenic assay and all contained < 0.01 IU/ml FVIII. There was a wide range in the amount of thrombin generated and the lag-time ($T_{½max}$) of the commercial plasmas (Fig 4.4, Table 4.2).

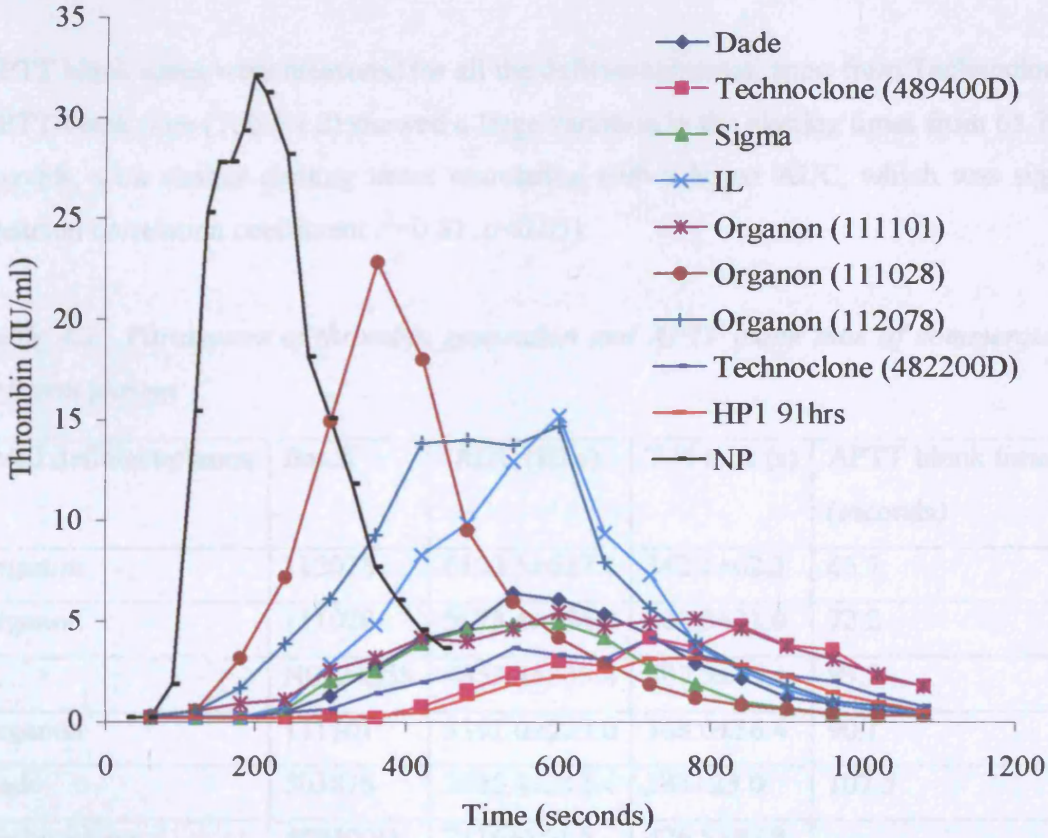


Fig 4.4. Thrombin generation by commercial FVIII deficient plasmas containing < 0.01 IU/ml FVIII.

There was a wide range in the amount of thrombin produced both between manufacturers and with Organon, between batches. The greatest amount of thrombin (AUC) was generated by Organon, which was 100% of normal plasma. The least amount was 33% which was generated by Technoclone. This is still considerably higher than that generated by haemophilic plasma at 91 hours post-infusion which generated 23% of normal plasma. Although a considerable amount of thrombin is generated in FVIII deficient plasmas the lag-time in all cases was prolonged when compared with normal plasma, and markedly prolonged in those FVIII deficient plasmas with a peak height of <7 IU/ml thrombin. There was no difference in the amount of thrombin generated by those plasmas which contained VWF (Organon, Technoclone, and Dade) and those which did not (Sigma and IL).

APTT blank times were measured for all the deficient plasmas, apart from Technoclone. The APTT blank time (Table 4.2) showed a large variation in the clotting times from 65.7 –107.5 seconds, with shorter clotting times correlating with a larger AUC, which was significant (Pearson correlation coefficient r^2=0.81, $p<0.05$).

Table 4.2. Parameters of thrombin generation and APTT blank time of commercial FVIII deficient plasma

FVIII deficient plasma	Batch	AUC (IU.s)	T ½ max (s)	APTT blank time (seconds)
Organon	112078	6102.5±627.1	342.1±62.3	65.7
Organon	111028	5673.4±584.8	280.0±21.0	72.2
IL	N0324258	4853.4±333.4	407.5±43.4	93.9
Organon	111101	3391.0±223.0	368.0±36.4	90.1
Dade	503875	2625.4±211.4	383±23.0	107.5
Technoclone	489400D	2116±164.5	476.5±87.8	-
Sigma	051K6030	2111.8±203.9	363.8±58.8	106.9
Technoclone	482200D	2019.3±69	574.8±72.8	106.4
NP		6069.0±410.5	118±9.0	-

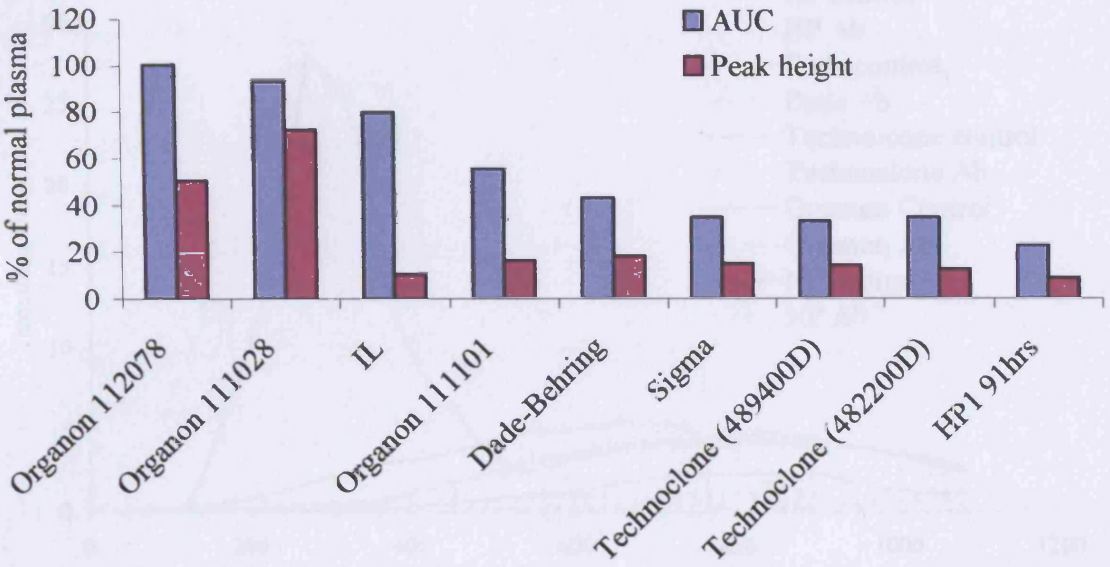


Fig 4.5 Area under the curve and peak height of FVIII deficient plasmas expressed as a percentage of normal plasma.

To establish if the observed thrombin generation was due to the presence of FVIII, normal plasma and commercial FVIII deficient plasmas were incubated overnight with a rabbit polyclonal anti-human FVIII Ab or a rabbit polyclonal anti-human growth hormone Ab as control. Plasma was incubated with polyclonal rabbit anti-FVIII antibody serum (kind gift of Dr Ingerslev) at a dilution of 1 in 1000 or polyclonal rabbit anti-human growth factor (kind gift of Dr Raferty, NIBSC) at a dilution of 1 in 1000. Samples were incubated overnight at 37°C, following incubation the plasma was defibrinated and thrombin generation measured as previously described.

Incubation with anti-FVIII Ab mostly abolished thrombin generation in all plasmas (Fig 4.6, Table 4.2). Thrombin generation was reduced in normal and haemophilic plasmas with lower AUC after treatment than in the commercial FVIII deficient plasmas. The reduction in AUC after treatment with FVIII Ab was not significant for Dade or the haemophilic plasma, but was significant for the other plasmas paired t-test $p<0.01$.

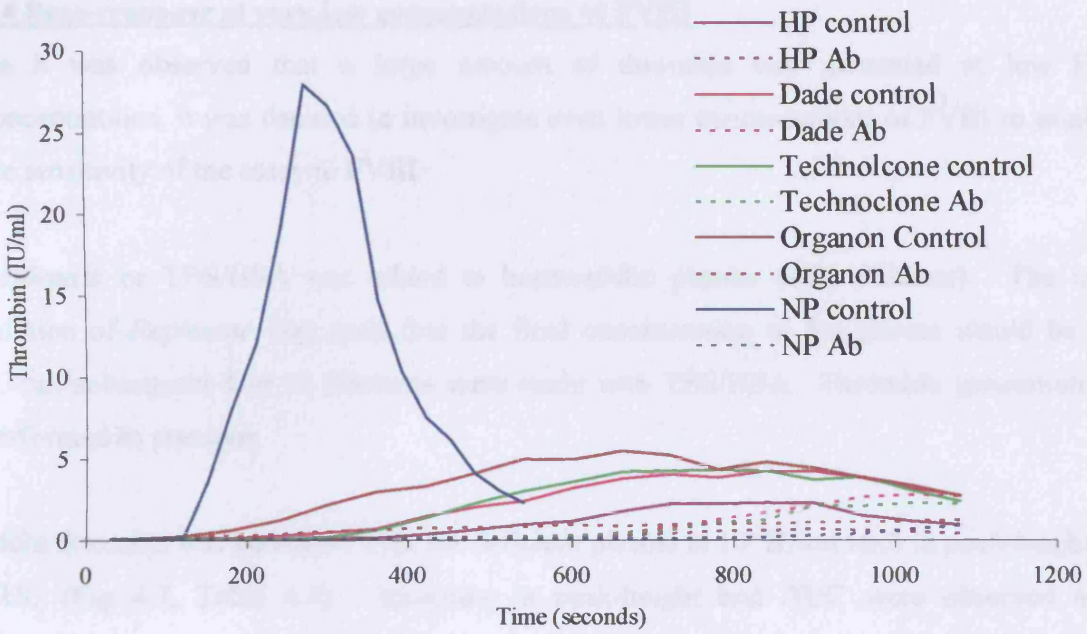


Fig 4.6. Thrombin generation of FVIII deficient plasmas after treatment with polyclonal anti-FVIII Ab or polyclonal growth hormone Ab ,following overnight incubation.

Table 4.3 AUC of FVIII deficient plasmas after treatment with polyclonal control Ab or polyclonal rabbit anti-FVIII Ab.

	HP 91	Dade	Organon	Technoclone	NP
Control Ab	1055±210.1	2706.7±301	3553.7±301	3012.1±1249.6	5134.5±624
Anti-FVIII Ab	315.9±271.9	760±876	811±26.2	1294.6±1344	218.94±97.7

As thrombin generation is reduced following treatment with an anti-FVIII antibody in all the plasmas tested, this would suggest that a small amount of FVIII is present in all commercial FVIII deficient plasmas.

4.4 Dose-response at very low concentrations of FVIII

As it was observed that a large amount of thrombin was generated at low FVIII concentrations, it was decided to investigate even lower concentrations of FVIII to establish the sensitivity of the assay to FVIII.

Replenate or TBS/HSA was added to haemophilic plasma (HP1 91hours). The initial dilution of *Replenate* was such that the final concentration in the plasma would be 0.01 IU/ml, subsequent 1 in 10 dilutions were made with TBS/HSA. Thrombin generation was performed as previous.

More thrombin was generated than the deficient plasma at 10^{-5}IU/ml both in peak-height and AUC (Fig 4.7, Table 4.4). Increases in peak-height and AUC were observed at all concentrations. However, these differences were only significant from blank at 0.001 IU/ml, $p<0.01$.

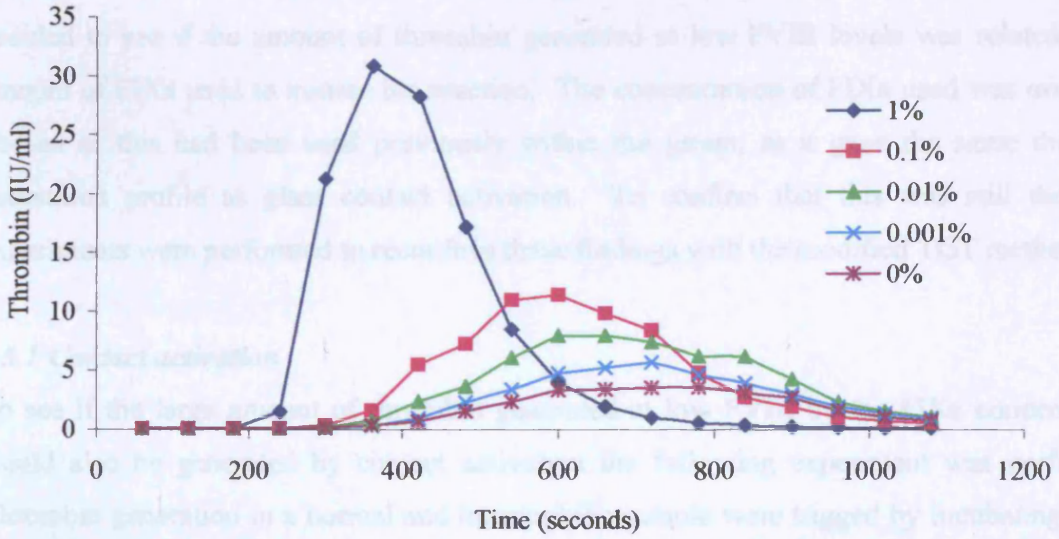


Fig 4.7. Thrombin generation test with very low levels of FVIII. Replenate or buffer was added to haemophilic plasma (HP1 91hours), triggered with high FIXa, PL and Ca^{2+}.

*Table 4.4 AUC and peak-height at low FVIII concentrations, only significantly different from blank at $1x10^{-3}$ IU/ml. *=$p<0.01$*

	10^{-2} IU/ml	10^{-3} IU/ml	10^{-4} IU/ml	10^{-5} IU/ml	0 IU/ml
AUC (IU.s)	6860.9±801.7 *	4089±915.7 *	3600.5±1174.7	2322.8±364.7	1700±541.6
Peak (IU/ml)	30.4±4.2 *	13.35±3.5 *	11.6±3.5	6.4±1.1	4.1±1.6

4.5 Effect of FIXa

The large amount of thrombin generated even at very low FVIII was an unexpected finding, especially due to the severe bleeding tendency of patients with <0.01 IU/ml FVIII. This would suggest that the assay may not represent what is occurring *in vivo*. This calls into question the concentration of FIXa used to trigger activation of the plasma. Therefore it was decided to see if the amount of thrombin generated at low FVIII levels was related to the amount of FIXa used to initiate the reaction. The concentration of FIXa used was originally chosen as this had been used previously within the group, as it gave the same thrombin generation profile as glass contact activation. To confirm that this was still the same experiments were performed to reconfirm these findings with the modified TGT method.

4.5.1 Contact activation

To see if the large amount of thrombin generated at low FVIII by the FIXa concentration would also be generated by contact activation the following experiment was performed. Thrombin generation in a normal and haemophilic sample were trigged by incubating 400µl plasma in a glass tube for 2 minutes followed by the addition of 400µl PL and 400µl Ca^{2+}, the rest of the reaction was carried out in the glass tubes.

Following contact activation the amount and time of thrombin generation was similar to that obtained with FIXa activation (Fig 4.8) in both plasmas. These findings suggest that the

amount of FIXa used to initiate the reaction is comparable to that obtained from activation via the intrinsic pathway.

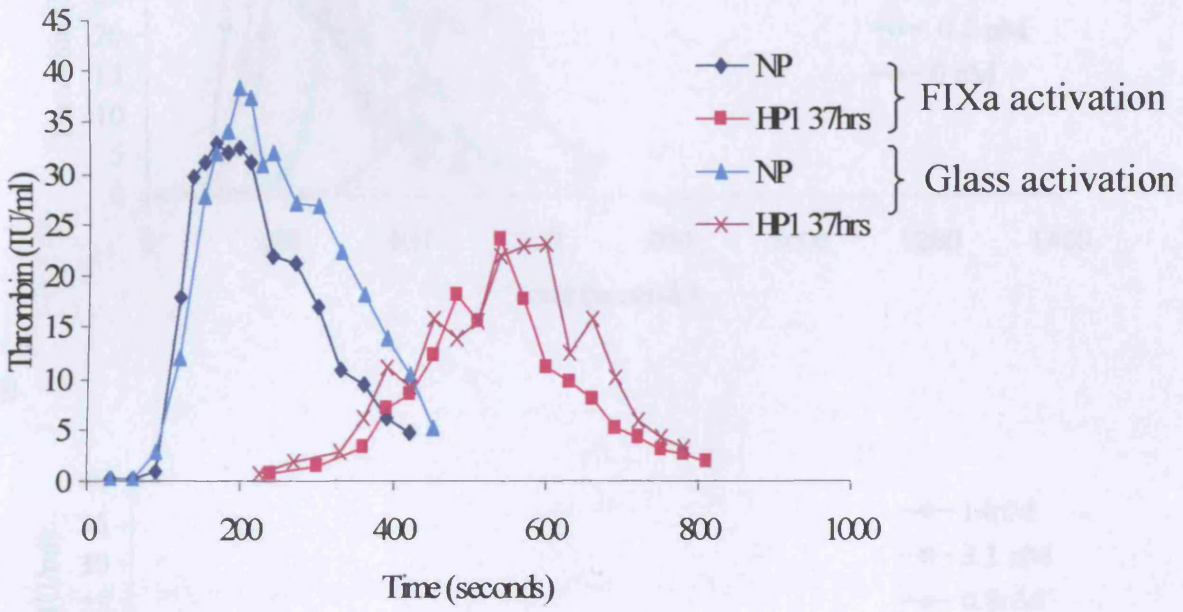
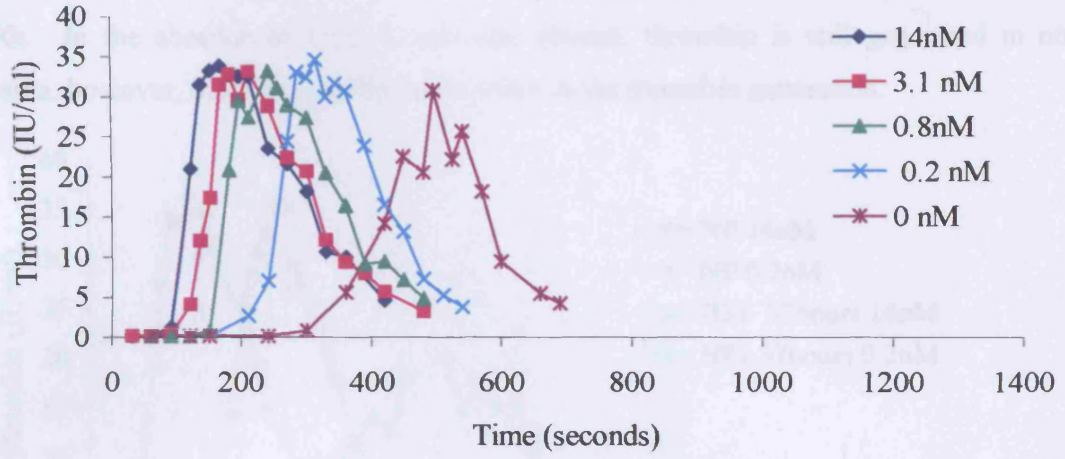


Fig 4.8 Comparison of thrombin generation by contact or FIXa activation for nomal and haemophilic plasma, n=1

4.5.2 Effect of decreasing concentration of FIXa on thrombin generation

As the concentration of FIXa used was comparable to that of contact activation the amount of FIXa was reduced to see what effect this had on thrombin generation at low FVIII concentrations.

A



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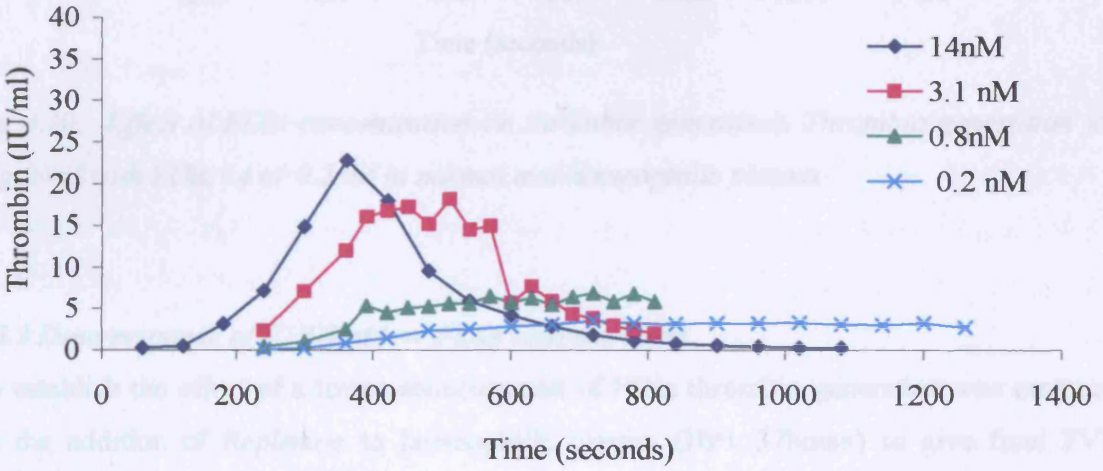


Fig 4.9 Thrombin generation at different concentrations of FIXa. A- Normal plasma B-FVIII deficient plasma (Organon) n=1.

The concentration of FIXa was attenuated (Fig 4.9) in order to check the influence of FIXa on thrombin generation at very low FVIII concentrations. Decreasing FIXa concentration from 14nM progressively prolonged the lag-time of normal plasma ($T_{½max}$ 114 seconds at 14nM to 226 seconds at 0.2nM) but caused no change in peak-height or AUC (at 0.2nM AUC 98% of 14nM). In contrast to results with FIXa concentration of 14nM, at 0.2nM in

haemophilic plasma, virtually no thrombin was generated. The thrombin generation test is therefore sensitive to the low amounts of FVIII but is dependent also on the concentration of FIXa. In the absence of FIXa to activate plasma, thrombin is still generated in normal plasma, however, there is a considerable delay in the thrombin generation.

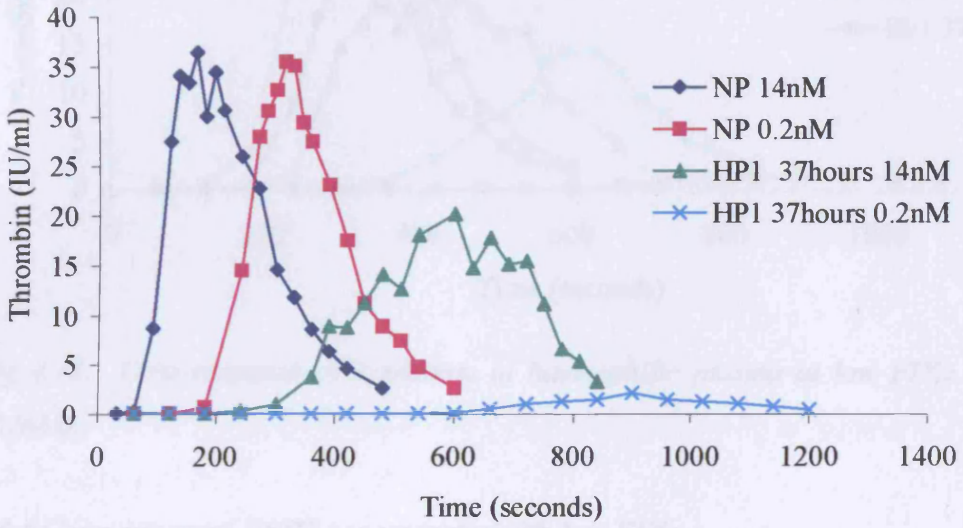


Fig 4.10. Effect of FIXa concentration on thrombin generation. Thrombin generation was triggered with FIXa 14 or 0.2nM in normal and haemophilic plasma.

4.5.3 Dose-response of FVIII at low FIXa concentration

To establish the effect of a lower concentration of FIXa thrombin generation was measured by the addition of *Replenate* to haemophilic plasma (HP1 37hours) to give final FVIII concentrations of 1-0.03 IU/ml. Thrombin generation was carried out as before using 0.2nM FIXa.

As FVIII concentration decreased, AUC and peak-height also decreased, and the lag-time increased (as was observed previously)(Fig 4.11). In contrast to the high FIXa concentration peak-height and AUC remained abnormal, and were only normalised by much higher FVIII concentrations. The effect on thrombin generation with increasing FVIII was more marked at a lower FIXa concentration.

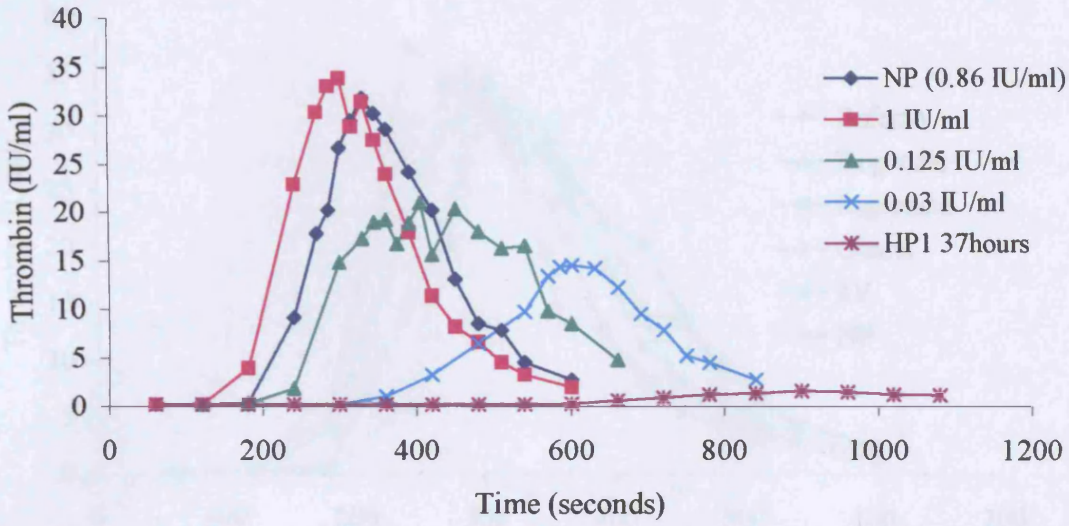


Fig 4.11. Dose-response of Replenate in haemophilic plasma at low FIXa concentration (0.2nM).

4.5.4 Comparison of FVIII concentrates with low FIXa

It was thought that the lower concentration of FIXa might enhance any differences between the FVIII concentrates that could not be readily observed between the concentrates as measured previously. Thrombin generation was measured for five different FVIII concentrates at a concentration of 1 IU/ml in haemophilic plasma (HP1 37hours).

The thrombin generation between the concentrates (Fig 4.12) was more variable than at the higher FIXa concentration (see section 3.6). *Refacto*, *Replenate*, *Octavi* and *8Y* all generated thrombin at around the same time with no difference in the $T_{½\ max}$ (ANOVA p=0.45). However, *Kogenate* was significantly slower to generate thrombin than the other concentrates (ANOVA p <0.001). With the exception of *Kogenate*, all concentrates generated thrombin more rapidly than normal plasma.

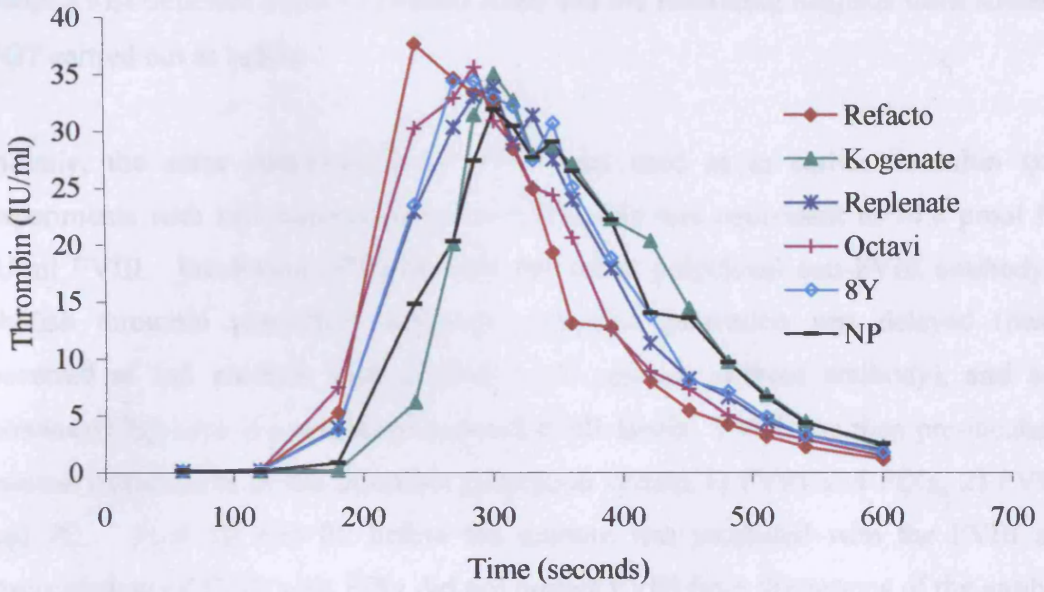


Fig 4.12 Thrombin generated by FVIII concentrates at 1 IU/ml with low FIXa activation concentration (0.2nM).

4.6 Protection from FVIII inhibitors by FIXa and PL

Previous experiments by Barrowcliffe *et al* (Barrowcliffe *et al*, 1983;Barrowcliffe *et al*, 1984) had demonstrated that incubation of FVIII with PL and FIXa protected FVIII from neutralisation by a high titre inhibitor plasma. The authors attributed this protection to the action of PL as FIXa excluded from the reaction mixture produced almost as much thrombin as when it was present. Since our results had shown that the FIXa concentration used has a marked effect on thrombin generation, it was decided to see if incubation of FVIII with FIXa had an additional protective effect to the use of PL alone. Our study differed in methodology to Barrowcliffe et al as they used a higher concentration of PL (25µg/ml) and undefibrinated inhibitor plasma.

Experiments were conducted by incubating 50µl *Hemofil-M* with one or more of the other reagents: 400µl PL (10 µg/ml), 400µl Ca^{2+}, 80µl FIXa (84nM), the complex was incubated for 5 minutes at 37°C, this was followed by a 30 min incubation with 50µl of polyclonal rabbit anti-FVIII antibody (01/460 20BU/ml) or 50µl of buffer. At the end of the incubation,

300µl FVIII deficient plasma (Technoclone) and the remaining reagents were added and the TGT carried out as before.

Initially, the same concentration of FIXa was used as in earlier thrombin generation experiments with low concentrations of FVIII, this was equivalent to 14.8 pmol FIXa per IU/ml FVIII. Incubation of FVIII with the rabbit polyclonal anti-FVIII antibody did not abolish thrombin generation, however, thrombin generation was delayed (peak-height occurred at 165 seconds with antibody, 120 seconds without antibody), and as shown previously lag-time is a marker of reduced FVIII levels. FVIII was then pre-incubated with various components of the thrombin generation system 1) FVIII and FIXa, 2) FVIII, FIXa and PL, 3) FVIII and PL before the mixture was incubated with the FVIII antibody. Preincubation of FVIII with FIXa did not protect FVIII from the actions of the antibody (Fig 4.13), as there was a slightly longer lag-time than FVIII alone. When FVIII was in complex with PL or PL combined with FIXa then thrombin was generated more rapidly than in the absence of the antibody i.e. thrombin generation was enhanced if FVIII was in complex with PL or PL and FIXa.

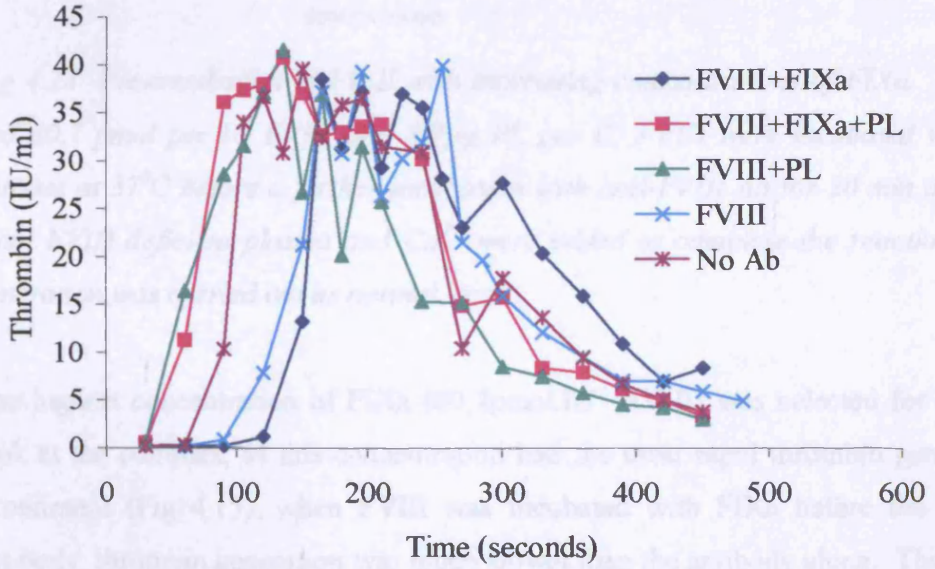


Fig 4.13 Protection of FVIII from a polyclonal anti-FVIII antibody by a 30 min preincubation of FVIII with FIXa, and PL.

To establish if the concentration of FIXa used was not optimal, further experiments were carried out with increasing concentrations of FIXa (14.8. 41.7 and 80.7 pmols.IU^{-1} FVIII). The same pattern was observed as previously i.e. that a more rapid thrombin generation was observed with the combined complex of FIXa and phospholipid.

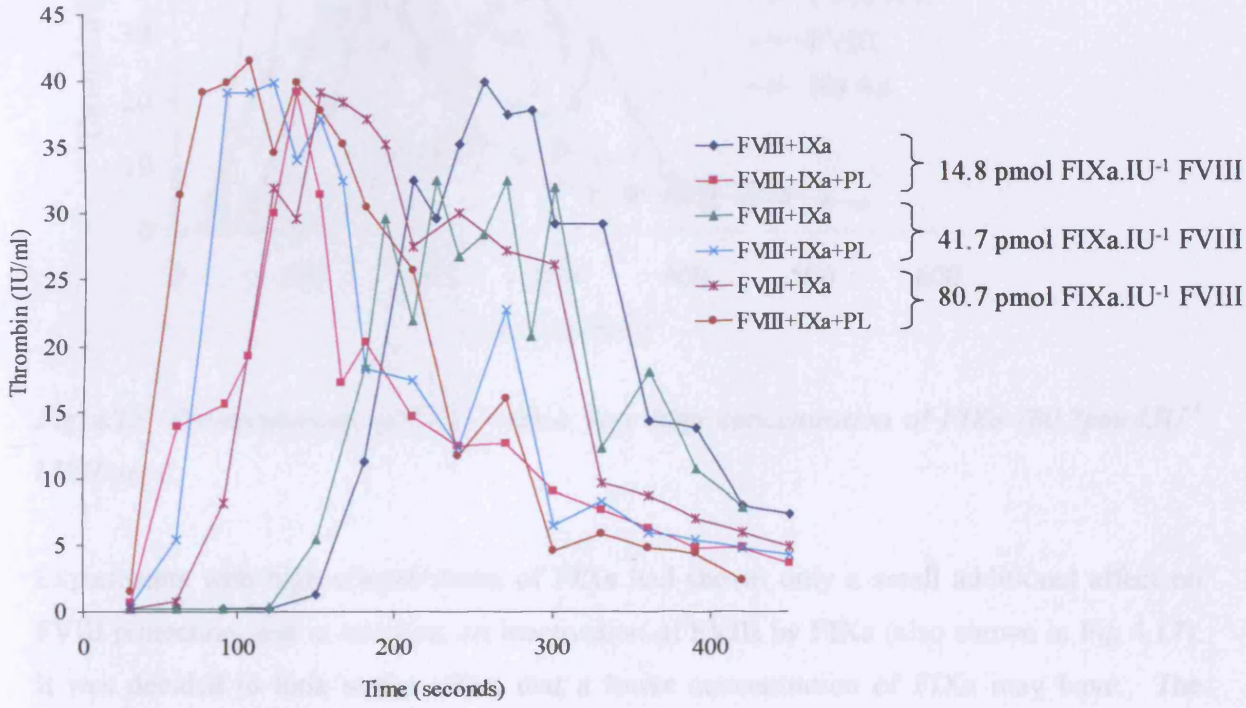


Fig 4.14 Pre-incubation of FVIII with increasing concentrations of FIXa. FIXa 14.8, 41.7 and 80.7 pmol per IU FVIII and 8.9µg PL per IU FVIII were incubated with FVIII for 5 minutes at 37^{0}C before a further incubation with anti-FVIII Ab for 30 min a 37^{o}C, at which point FVIII deficient plasma and Ca^{2+} were added to complete the reaction and thrombin generation was carried out as normal. n=1

The highest concentration of FIXa (80.7pmol.IU^{-1} FVIII) was selected for a more detailed look at the complex, as this concentration had the most rapid thrombin generation. In this experiment (Fig 4.15), when FVIII was incubated with FIXa before the addition of the antibody, thrombin generation was much slower than the antibody alone. This would suggest that at this concentration FIXa is inactivating FVIII. The combined complex of FIXa and phospholipid did offer more protection from the antibody than PL alone, although this was not a marked finding.

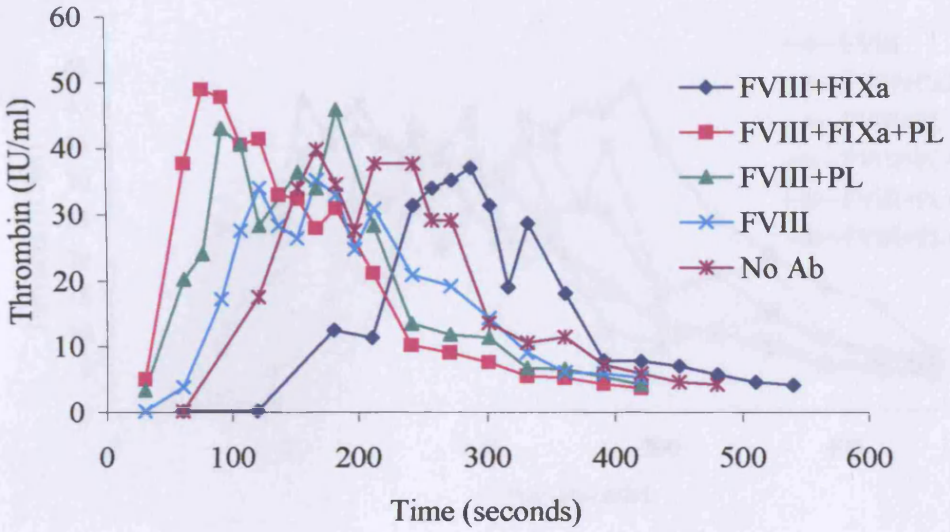
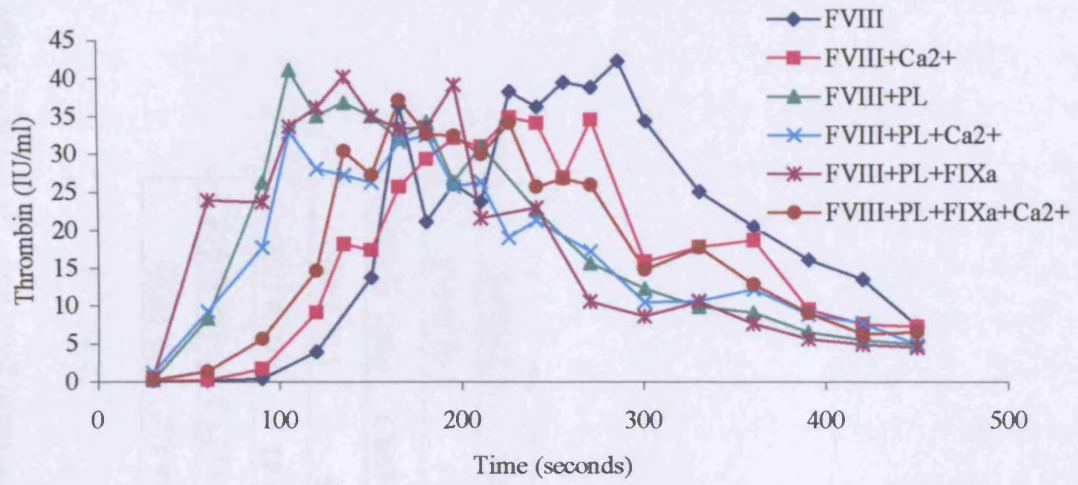


Fig 4.15 Pre-incubation of FVIII with a very high concentration of FIXa (80.7pmol.IU^{-1} FVIII) n=1.

Experiments with high concentration of FIXa had shown only a small additional affect on FVIII protection, and in addition, an inactivation of FVIII by FIXa (also shown in Fig 4.17). It was decided to look at the effect that a lower concentration of FIXa may have. The concentration that produced minimal thrombin generation in FVIII deficient plasma was chosen. In addition calcium was also added to the various complexes to assess what affect this may have on thrombin generation (Fig 4.16).

A



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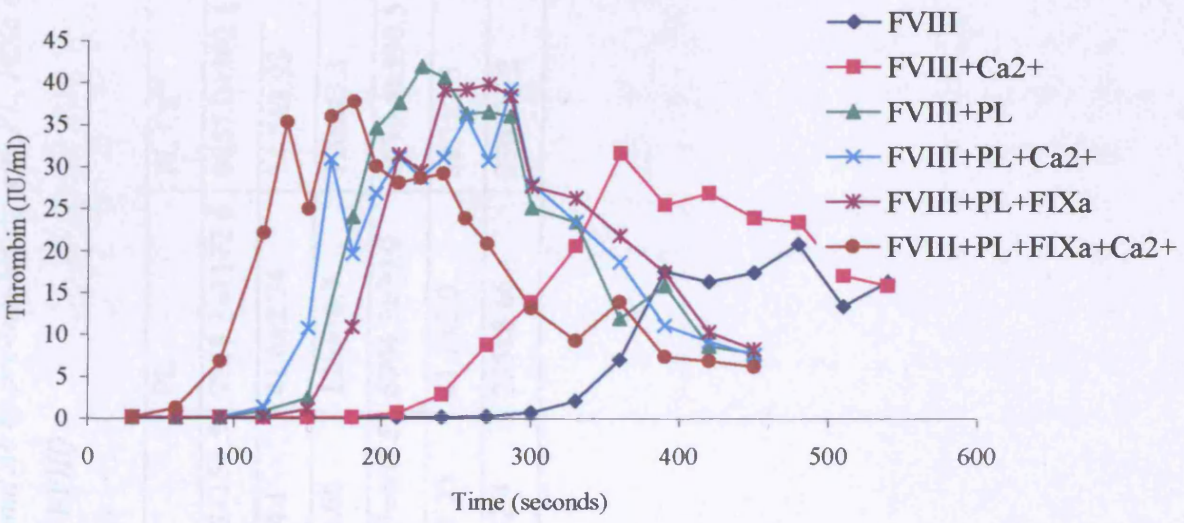


Fig 4.16 Protection of FVIII by PL, IXa and Ca^{2+}. FVIII was incubated with PL, IXa or Ca^{2+} before a 30 min incubation with rabbit polyclonal anti-FVIII antibody. The remaining reagents for thrombin generation were added at the end of the incubation and thrombin generation measured. A) Protection by High IXa (14.8pmol.IU^{-1}FVIII) B Protection by low IXa (0.23pmol.IU^{-1} FVIII).

Table 4.5 Protection of FVIII from a polyclonal Ab by pre-incubation with PL, FIXa or Ca^{2+}-thrombin generation parameters. High IXa (14.8pmol.IU^{1}FVIII, low IXa (0.23pmo.IU^{1} FVIII).

		Control	Ca^{2+}	PL	PL Ca^{2+}	PL FIXa Ca^{2+}	PL FIXa
High IXa	AUC	7605.1±706	6752.8±154.5	7818.3±1172.6	6857.0±302.1	6890.3±155.3	7402.3±658.5
	Peak	40.0±2.0	36.5±4.1	41.6±2.74	34.7±3.93	38.3±0.43	40.0±1.72
	Time	260±31.2	200±8.66	125±18.7	190±43.3	180±15	116.3±28.4
Low IXa	AUC	3130.1±2087.6	6249.9±664.5	6994.3±70.0	6679.4±590.5	6655.0±99.3	6901.9±230.2
	Peak	20.1±13.7	34.0±3.25	41.3±2.0	38.1±2.5	37.9±3.1	42.0±0.5
	Time	440±62.4	380±62.4	235±8.66	230±31.2	200±34.6	270±15

Incubation of FVIII with the polyclonal antibody did not abolish thrombin generation even at this low FIXa concentration, as the incubation period was not long enough to achieve this. However, the lag-time was increased at both concentrations of FIXa. Incubation of FVIII with Ca^{2+} alone did offer some protection from the antibody with a reduction in the lag-time. However, this finding was not significant for either concentration of FIXa ($p=0.07$ and 0.3, for high and low concentrations respectively). Maximum protection was observed with the high FIXa concentration with PL; PL, IXa; and PL, Ca^{2+}. The combined complex of FVIII, PL, IXa and Ca^{2+} was significantly less effective than the complex without Ca^{2+} ($p< 0.05$). This was in contrast to the findings with the lower FIXa concentration in which the most effective protection of FVIII from Ab was by PL, IXa and Ca^{2+}, although in these experiments this was not significantly different from PL and IXa ($p= 0.06$).

The results above suggested that the high FIXa concentration was producing a detrimental effect on FVIII compared to the lower FIXa concentration in which FIXa increased the protective effect against the FVIII antibody. To clarify this *Hemofil-M* was incubated with either concentration of FIXa for 30 minutes or with no pre-incubation and thrombin generation carried out as normal (i.e. in the absence of antibody). The high FIXa concentration caused a delay in thrombin generation when FVIII was incubated with this concentration for 30 minutes (Fig 4.17). A delay in the lag-time was not observed with the lower concentration of FIXa and suggests that the higher concentration is causing inactivation of FVIII.

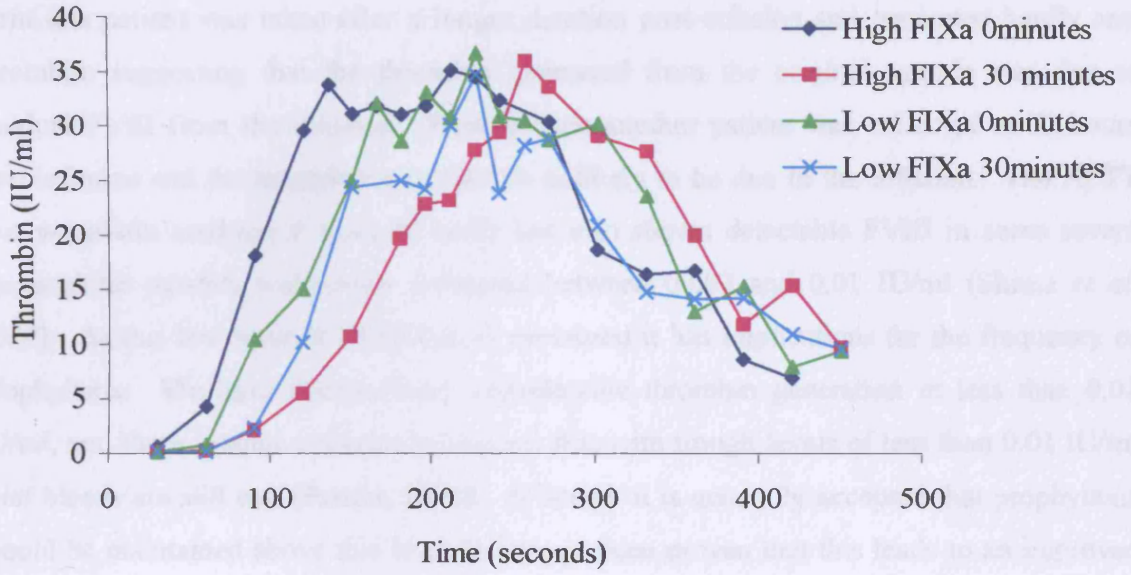


Fig 4.17 Effect of incubation time on thrombin generation following incubation of FVIII with FIXa. High FIXa (14.8pmol.IU^{-1}FVIII) low FIXa (0.23pmo.IU^{-1} FVIII). (n=2)

4.7 Discussion

With a high FIXa concentration the TGT was found to be very sensitive to low levels of FVIII. A large amount of thrombin was generated when FVIII levels were less than 0.01 IU/ml in both an artificial situation in which FVIII concentrate was added to FVIII deficient plasma, and in two severe haemophilic plasmas. Early work on the TGT found that only very small amounts of thrombin could be detected in haemophilic plasma, however, only small additions of normal plasma were required to improve the thrombin generation. Macfarlane and Biggs in their plasma studies showed that an addition of 0.5% normal blood to haemophilic blood caused detectable thrombin generation, although, it was prolonged and was only one third of the average peak height of the normal samples (Macfarlane & Biggs, 1953). Pitney and Dacie found that addition of 1% normal plasma improved thrombin generation, but that 20% or more was required to bring the thrombin generation within the

normal limits (Pitney & Dacie, 1953). The use of a FVIII antibody on a haemophilic sample demonstrated that thrombin generated below 0.01 IU/ml was due to FVIII. A repeat sample from this patient was taken after a longer duration post-infusion and generated hardly any thrombin, suggesting that the thrombin generated from the original sample was due to residual FVIII from the infusion. Plasma from another patient was collected at 72 hours post-infusion and the thrombin generated is unlikely to be due to the infusion. The APTT clot waveform analysis in a recent study has also shown detectable FVIII in some severe haemophilic patients with levels measured between 0.002 and 0.01 IU/ml (Shima *et al*, 2002). As this low level of FVIII can be measured it has implications for the frequency of prophylaxis. We have demonstrated considerable thrombin generation at less than 0.01 IU/ml, and there is some evidence to suggest that with trough levels of less than 0.01 IU/ml joint bleeds are still rare (Petrini, 2001). Although it is generally accepted that prophylaxis should be maintained above this level, it has not been proven that this leads to an improved clinical outcome (van den Berg *et al*, 2001).

The sensitivity of the TGT to low levels of FVIII may be explained by the amount of FIXa used in the assay to initiate coagulation. It was found that the FIXa concentration was important in the ability of low concentrations of FVIII to generate thrombin, but this effect was minimised as levels of FVIII normalised. The high FIXa concentration was originally chosen as it produced the same thrombin generation profile as intrinsic glass contact activation. The amount of thrombin generated in plasma is controlled by the amount of FIX present as well as FVIII. At low TF concentrations, FIX is activated by the FVIIa-TF complex, which explains the role of the anti-haemophilic factors in thrombin generation (Østerud & Rapaport, 1977;Bauer, 1997;Keularts *et al*, 2001b). In addition, at low concentrations of TF, less than 1% of total plasma FIX is activated, which would give a plasma FIXa concentration of 0.9nM (Lawson *et al*, 1994). The low FIXa concentration of 0.2nM that has been used in these experiments is therefore lower than this, but could be considered to be a physiological concentration present during initiation of the coagulation cascade. The higher FIXa concentration of 14nM used to trigger thrombin generation, although similar to contact activation, would perhaps appear to be higher than physiological, although local concentrations of FIXa following injury may be higher than 0.9nM. Other

work has used FIXa concentrations of 0.1nM to 1.5nM in the TGT (Hoffman *et al*, 1996;Jetsy, 1991;Kumar *et al*, 1994;Sekiya *et al*, 1996). It may be that at high FIXa concentrations when assaying low concentrations of FVIII the FIXa protects what little FVIIIa is produced by the haemophilic plasma from degrading (Lenting *et al*, 1998), and at much higher FIXa concentrations FIXa can enhance FVIII activity (Rick, 1982). These high FIXa conditions in the TGT maybe artificial, but could be useful in practical terms for detecting low levels of FVIII, for example in gene therapy.

In gene therapy clinical trials to date, there have been some individuals with a reduced need for FVIII despite no observed increase in FVIII levels (White, 2001). The TGT may be better suited to detecting small increases in FVIII levels under these circumstances. The TGT may also be able to shed light into a minority of patients (10-15%) with FVIII of less than 0.01 IU/ml but with a clinical status that is better than the FVIII level suggests. It has been suggested that the most common prothrombotic mutation, factor V_{Leiden} with a carrier rate of 8.8% in Europeans (Rees *et al*, 1995) and 6% in white North Americans (Ridker *et al*, 1995), may account for those with a less severe clinical phenotype among patients with the same FVIII mutation (Nichols *et al*, 1996), and children with prothrombotic mutations develop their first bleed significantly later than those without (Ettingshausen *et al*, 2001). An *in vitro* model has also shown increased thrombin formation with the factor V_{Leiden} mutation (van't Veer *et al*, 1997). However, most haemophilic patients with factor V_{Leiden} still have a severe clinical outcome (Lee *et al*, 2000).

At lower concentrations of FIXa, the dose-response produced by a high-purity concentrate was more marked. Under these conditions all the parameters of the TGT were affected. As concentration of FVIII decreased, $T_{½max}$ increased as for the high FIXa concentration, but in contrast the peak height and the AUC decreased at the lower FIXa concentration. This version of the TGT may therefore be more useful for monitoring patients' response after infusion of concentrates.

The clotting method has shown a great sensitivity towards FVIII with the ability to distinguish levels less than 0.01 IU/ml. This was an unexpected finding as the detection limit

of the chromogenic and one-stage APTT FVIII assays are carried out, in general on automated machines, which have a higher precision than manual methods. The TGT could be readily used by laboratories which do not have expensive machinery as it is performed on a relatively simple machine which records the time to clot. The TGT can also be performed by hand with tubes in a water bath. The other advantage of the TGT as a valuable technique for laboratories in developing countries is the cost of reagents which are also low.

The sensitivity of the TGT to low levels of FVIII could be useful in the measurement of prophylaxis. We did examine a small cohort of children receiving prophylaxis treatment. There was no significant correlation between the AUC, peak-height or time-to-peak and the measured FVIII level. However, the TGT does provide additional information that the FVIII level alone cannot provide. The peak-height and time-to-peak are important parameters, in that, the rate at which thrombin is generated is probably more important than the total amount of thrombin produced. One sample generated no thrombin despite a measured FVIII level of 3 IU/ml, near normal thrombin generation would have been expected with this FVIII level. This therefore suggests contamination of the sample, as the patient had a central line, heparin would be the most likely cause.

The TGT may also be a useful tool for shedding light on those patients with different phenotypes. It has been known for some time that there is a subset of severe patients in whom spontaneous bleeding is less common. As the current diagnostic assays are only sensitive to 0.01 IU/ml, this level is the definition for severe haemophilia. However, some patients may have a very small amount of endogenous FVIII, which is not detected by the current assays. Recent work has demonstrated that there are patients with severe haemophilia who fall into two distinct groups. By measuring the clot waveform analysis Shima et al demonstrated that two thirds of patients had FVIII levels between 0.002-0.01 IU/ml, while the remaining patients had levels less than 0.002 IU/ml (Shima *et al*, 2002). In addition Ingerslev et al have shown that severe patients have two different types of ro-TEG profiles. They also found a difference in the amount of FVIII required to normalise the velocity of coagulation, with some patients requiring 0.05 IU/ml, whilst for others an order of magnitude greater was required (Ingerslev *et al*, 2003). The differences between those

severe haemophilacs which regularly bleed "severe severe" and those that do not "moderate severe" is currently being investigated by the SSC of the ISTH. The results presented here would also suggest that the TGT triggered with the high FIXa concentration may also be able to differentiate between the two different types of patient. However, despite recent developments in assays which are more sensitive to low levels of FVIII the current ISTH definition for severe haemophilia (<0.01 IU/ml) is not being adhered to and there is widespread misunderstanding amongst clinical laboratories in diagnosis and in ability to reliably measure samples containing < 0.01 IU/ml (Preston *et al*, 2004). This would suggest that further definitions would not be practical at this time.

Studies described above with FVIII deficient plasmas have shown a wide variation in the amount of thrombin generated and a considerable variation between the batches of one product. Great care must be used to find an appropriate FVIII deficient plasma for use in the TGT. The choice of FVIII deficient plasma may also have a bearing on the one-stage APTT assay as there is a reliance on deficient plasma in this method. In particular the large amounts of thrombin generated by some plasmas, may limit the sensitivity of the assay to low levels of FVIII. The choice of FVIII deficient plasma was also shown to be important in the Bethesda assay for measuring FVIII inhibitors (Verbruggen *et al*, 2001). This study also found that chemically depleted plasma contained FVa, leading to over estimation of inhibitor titres. It would seem likely that the rapid generation of thrombin which occurred in our assay with chemically depleted plasma was due to the presence of FVa, as FVa can increase the rate of thrombin formation by 12,900 fold (Philippou *et al*, 1996).

Previous work has shown that PL can protect FVIII from inhibitors (Barrowcliffe *et al*, 1983). These authors also looked briefly at FIXa in the complex but at a FIXa concentration of 1μg/ml to 1 IU/ml FVIII, FIXa did not improve the protection by PL alone. This is in agreement with the results presented here as the concentration used by Barrowcliffe et al is approximately 15pmol FIXa.IU^{-1} FVIII. We investigated if FIXa would add to this affect as thrombin generation is increased by FIXa. High concentrations of FIXa were initially used, but this was found to be detrimental to FVIII, presumably by inactivation of FVIII by FIXa (O'Brien *et al*, 1992). The most effective concentration of FIXa for protection of FVIII was

found to be a much lower concentration of 0.2pmol.IU^{-1}FVIII (0.2nM), which was the same concentration that gave minimal thrombin generation in FVIII deficient plasma. This concentration of FIXa was most effective when Ca^{2+} was included in the complex, although this was not a significant improvement on the complex without calcium. In addition calcium also offered some protection from the polyclonal antibody on its own. Calcium ions are known to stabilise FVIII (Mikaelsson *et al*, 1983) and this may be occurring, or the calcium ions may be preventing the antibodies from binding to FVIII by blocking binding sites or altering the conformation. The protection of FVIII against inhibitors by calcium ions has also been observed in a clinical trial, when patients were infused with calcium before or during FVIII replacement a greater reduction in inhibitor titre was observed (Muhleman *et al*, 1985). This combined approach could be a significant finding, as an inhibitor to FVIII is a major hindrance to treatment and quality of life.

4.8 Summary

This chapter presents results in which it is demonstrated that the thrombin generation test is very effective at measuring low FVIII levels < 0.01 IU/ml which is below the detection limit of conventional FVIII assays. The TGT has also shown great versatility in measurement both at high and low FVIII concentrations, which has not been previously demonstrated.

The amount of thrombin that is generated by these low FVIII levels is potentiated by the FIXa concentration used to initiate thrombin generation. With the original FIXa concentration large amounts of thrombin (two-thirds of normal plasma) can be generated even by severe haemophilic plasmas, however if the level of FIXa is attenuated, the amount of thrombin generated is also reduced. The sensitivity of the thrombin generation test has several implications for management of haemophilic patients. The thrombin generation test could be a useful tool for monitoring low FVIII levels, for example in prophylaxis, phenotype monitoring and for measuring gene therapy.

CHAPTER 5 FLUOROGENIC THROMBIN GENERATION TEST

5.1 Introduction

This section describes results obtained with the fluorogenic TGT. This method was chosen for several reasons: reduced sample volume; the fluorogenic substrate is unaffected by turbidity, therefore platelets can be added to un-defibrinated plasma for a closer approximation to the *in vivo* situation; also it is a method which does not require subsampling.

There are several methods by which thrombin generation can be measured, clotting of fibrinogen, subsampling into a chromogenic substrate and continuous measurement using a slow chromogenic or fluorogenic substrate. The continuous fluorogenic substrate method has been developed by Hemker's group (Hemker *et al*, 2000) to overcome the limitations of chromogenic methods. The chromogenic methods are affected by the turbidity of the plasma samples and as a consequence, samples must first be defibrinated, and PRP must be studied by a labour intensive subsampling methodology. Defibrinated samples are thought to be unphysiological, as it has been postulated that the presence of fibrin in PRP may activate platelets (Béguin & Kumar, 1997). Therefore, the fluorogenic method allows thrombin generation to be determined in a more physiological system and without the need for subsampling to be hindered by removal of the developing clot. An additional advantage of continuous thrombin generation is that the method can be carried out in a microtitre plate minimising the volume of plasma required, and allowing more TGTs to be performed.

The main disadvantage of the continuous registration of thrombin with a fluorogenic or chromogenic substrate is that α2-macroblobulin forms a complex with thrombin which also cleaves the substrate. The α2-macroglobulin-thrombin contribution can be subtracted with the use of a mathematical equation (Hemker *et al*, 1993), as detailed in the material and methods section (2.3.2). Another disadvantage is that thrombin generation is measured in terms of relative flouresence units (RFU), as opposed to the other tests which can be

converted to thrombin. Hemker's group has more recently developed a calibrator for conversion of the RFU to thrombin (Hemker *et al*, 2003).

5.2 Reproducibility of fluorogenic assay

Initial experiments were performed with this method to test the reproducibility and to establish if any wells needed to be excluded on the microtitre plate. Normal plasma was added to each well of the microtitre plate and thrombin generation performed as described in the methods section (2.2.3). In brief, 40µl of non-defibrinated plasma was placed in a well of a black microtitre plate. To this was added 80µl of the remaining reagents FIXa, PL, Ca^{2+} and the substrate Z-gly-gly-arg-pro. Final reaction concentrations were plasma 33%, FIXa 5nM, PL 3µg/ml, Ca^{2+} 7mM, Z-gly-gly-arg-pro 0.234 nM. Thrombin generation was measured by excitation at 390nm and emission at 460nm at 30 second intervals for one hour. These experimental conditions were used throughout unless mentioned otherwise, samples were run in each assay in duplicate and assays were repeated three times (unless otherwise stated)

The fluorogenic assay used here was designed to be similar to the clotting method i.e. plasma constituted a similar proportion of the reaction mixture and reagents were also utilised at a similar final concentration (Table 5.1). The similar design between the two methods would allow for some comparisons between the methods.

Table 5.1 Comparison of final reagent concentrations in both TGT methods.

	Clotting	Fluorogenic
Plasma	30%	33%
PL	3.1µg/ml	3 µg/ml
Ca^{2+}	7.8 mM	7mM
FIXa	5.2 nM	5nM
Z-gly-gly-arg-pro	-	0.234 nM

The intra-assay variability gave a CV of between 3.8 and 9.5% (Table 5.2). The data was analysed by Mr Peter Rigsby (NIBSC) and all surrounding wells were excluded as was row F due to high varability in these wells. Exclusion of these wells reduced the intra-assay variability to 1.7-3.8% (Table 5.3). All subsequent experiments were performed excluding the use of these wells for samples, with the inclusion of buffer in all empty surrounding wells.

Table 5.2. Reproducibility of AUC. Normal plasma was assayed in each well of a 96 microtitre plate n=4.

	1	2	3	4
Mean	45583.53	43939.88	45182.81	44913.07
SD	2403.428	4178.102	1813.33	1706.15
CV	5.3	9.5	4.0	3.8

Table 5.3. Reproducibility of AUC with exclusion of wells. Columns 1 and 12 and rows A, F and G of a 96-well microtitre platewere excluded from data in table 5.2 n=4.

	1	2	3	4
Mean	47279.95	46265.91	46597.91	46186.5
SD	1755.2	1745.6	772.9	830.2
CV	3.8	3.8	1.7	1.8

The inter-assay variability was also assessed for normal pooled plasma and *Replenate* dilutions in Technoclone FVIII deficient plasma (Table 5.4). The largest variation was observed for peak-height, which ranged from 8.7-14.1%. The least variable parameter was the time-to-peak which varied by 4.4-8.8%. In addition, thrombin generation by normal plasma was more variable (6.4-14.1%) than that produced by the low FVIII concentrations, for example the FVIII deficient plasma varied by 4.3-8.7%.

Table 5.4. Reproducibility fluorogenic TGT with different FVIII concentrations. Normal plasma, Technoclone and dilutions of normal plasma in Technoclone were assayed, n = 5.

	AUC (RFU)		%	Peak (RFU)		%	Time to peak (min)		%
	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
NP	43615.3	5674.2	13	3125.7	441.8	14.1	10.15	0.65	6.4
0.03 IU/ml	37673.0	785.1	2.1	2891.0	213.2	7.4	11.85	1.0	8.8
0.008 IU/ml	36142.8	2133.7	5.9	2721.2	329.4	12.1	13	1.0	7.4
Technoclone	29490.9	1269.2	4.3	1485.6	129.9	8.7	22.0	1.0	4.4

5.3 Effect of fibrinogen

A comparison was carried out between the fluorogenic and clotting methods of thrombin generation at different FVIII concentrations in both defibrinated and non-defibrinated plasmas.

To remove the fibrinogen, plasmas were treated with ancrod (0.5 IU/ml) for 30 minutes at 37°C. The plasmas were prepared at the same time and measured by each method simultaneously. In the clotting method, in samples which were not defibrinated the clot was removed by winding the clot onto a wooden stick as it formed, the stick was then pressed against the side of the tube in order to remove the plasma from the clot. The wooden stick with the fibrin attached was left in the reaction mixture.

5.3.1 Clotting method

In the clotting method the AUC was higher when the samples were defibrinated 9032.1±653.5 IU/ml.s compared to 5225.2±638 in non-defibrinated plasma, ($p<0.05$) (Fig 5.1, Table 5.5). The peak-height was also significantly higher in defibrinated plasma for

normal plasma 25.5±2.6 IU/ml compared to 48.3±2.1 IU/ml in non-defibrinated plasma, ($p<0.05$). However, removal of fibrinogen did not have any effect on the time to peak height (185±34.6 seconds in non-defibrinated plasma, 172.5±22.5 seconds in defibrinated normal plasma). These differences were also found both in deficient plasma and with an addition of 3% normal plasma to FVIII deficient plasma (Technoclone).

Table 5.5. Comparison of TGT parameters with and without fibrinogen in the clotting system. NP, the addition of 3% NP to Technoclone, and Technoclone alone.

		Non-defibrinated			Defibrinated		
		NP	3%	Technoclone	NP	3%	Technoclone
AUC	Mean	5225.2	6071.6	1134.8	9032.1	9721.9	4355.4
(IU/ml.s)	SD	638.0	182.7	79.5	653.5	715.9	1341.4
Peak	Mean	25.5	28.0	3.0	48.3	48.1	11.6
(IU/ml)	SD	2.6	1.6	0.5	2.1	4.3	3.0
Time	Mean	185	375	890	172.5	320	670
(s)	SD	34.6	54.1	121.2	22.5	31.2	45.8

From the graph (Fig 5.1) it can also be seen that the peak of thrombin generation is maintained for a longer duration in the non-defibrinated samples compared to defibrinated plasma.

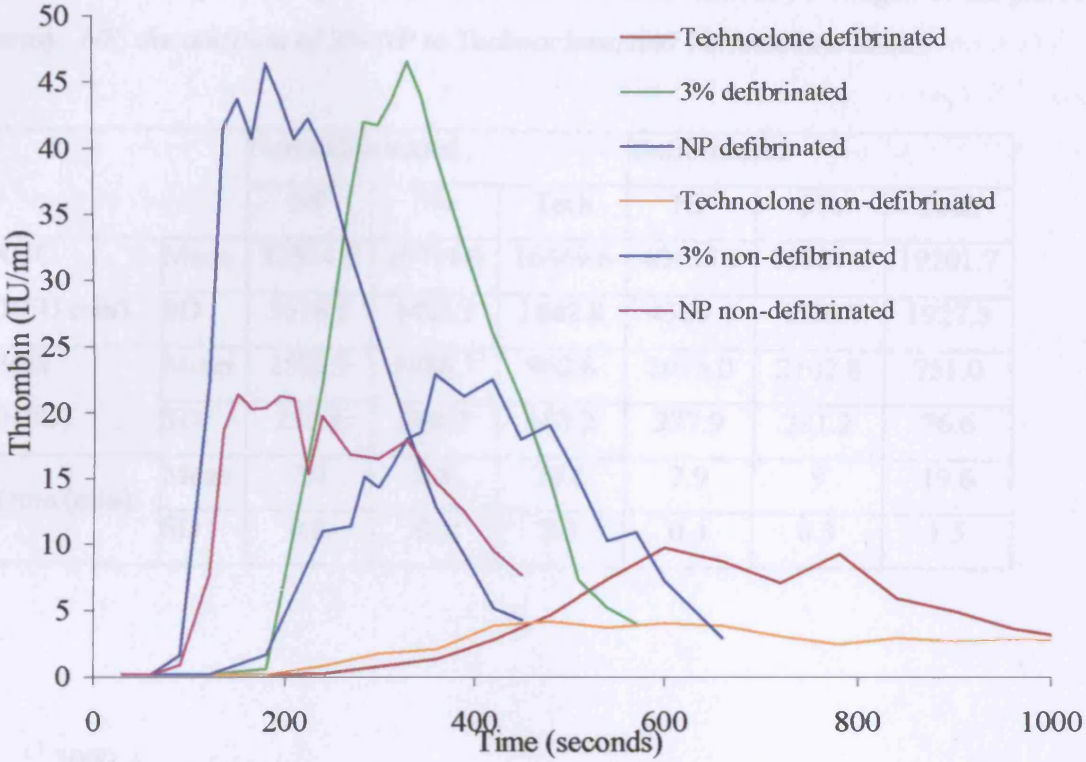


Fig 5.1. Effect of fibrinogen on thrombin generation in the clotting assay. Comparison of NP, 3% NP and Technoclone, all with and without defibrination.

5.3.2 Fluorogenic method

With the fluorogenic method, a significantly higher AUC was also obtained in defibrinated normal plasma (Fig 5.2, Table 5.6), 32565± 3617 compared with non-defibrinated 40405±4369 RFU.min ($p<0.05$). However, the increase in AUC was not due to an increase in peak-height, but due to sustained thrombin generation as the curves did not return to the baseline. The differences in peak-height between non-defibrinated and defibrinated were only significant with 3% normal plasma which would suggest a trend towards reduced peak. The fibrinogen did not affect the time-to-peak for the samples when measured with the fluorogenic method.

Table 5.6. Comparison of TGT parameters with and without fibrinogen in the fluorogenic assay. NP, the addition of 3% NP to Technoclone, and Technoclone alone.

		Non-defibrinated			Defibrinated		
		NP	3%	Tech	NP	3%	Tech
AUC	Mean	32564.5	26711.0	16469.6	40405.2	36881.1	19201.7
(RFU.min)	SD	3616.5	3425.5	1842.8	4369.4	4536.7	1927.5
Peak	Mean	2565.9	2488.5	962.6	2095.0	2102.8	751.0
(RFU)	SD	259.8	284.0	163.2	277.9	281.2	76.6
Time (min)	Mean	7.0	9.5	20.8	7.9	9	19.6
	SD	0.8	0.4	1.5	0.4	0.3	1.5

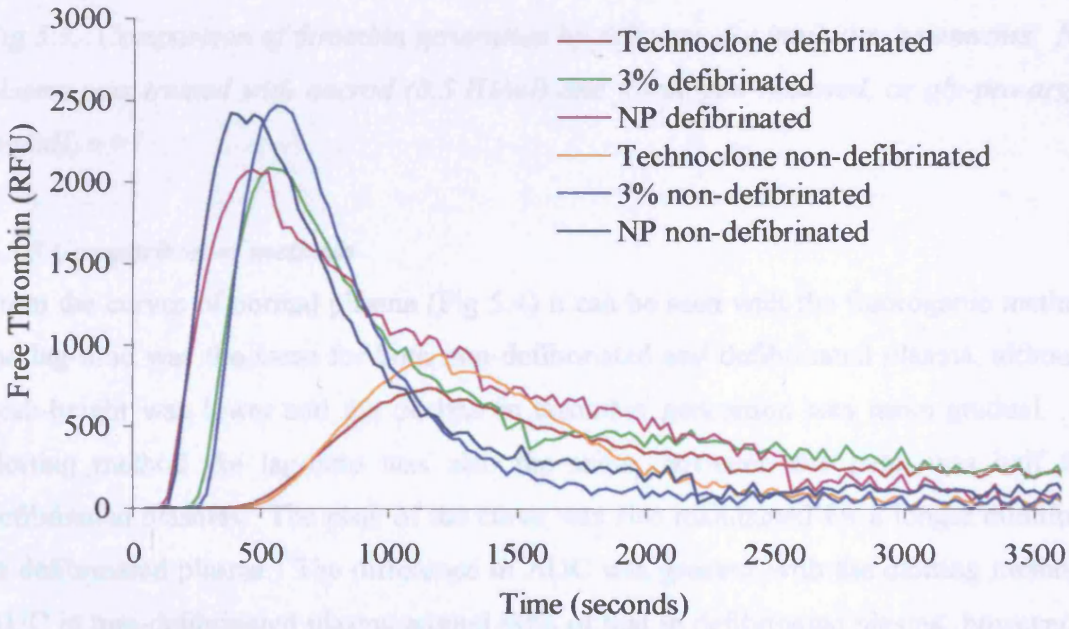


Fig 5.2. Effect of fibrinogen on thrombin generation in the fluorogenic assay.

To determine if the differences in the thrombin generation profile were due to the absence of fibrin, plasma was treated with the peptide gly-pro-arg-pro (4mg/ml plasma) which is an

inhibitor of fibrin polymerisation (Pratt *et al*, 1997;Laudano & Doolittle, 1980). Plasma treated with the peptide (AUC 56580 RFU.min) behaved in the same way as ancrod treated plasma (AUC 54952 RFU.min), whereas untreated plasma had a lower AUC of 41626 RFU.min (Fig 5.3). This would suggest that the absence of polymerised fibrin caused the increase in thrombin generation.

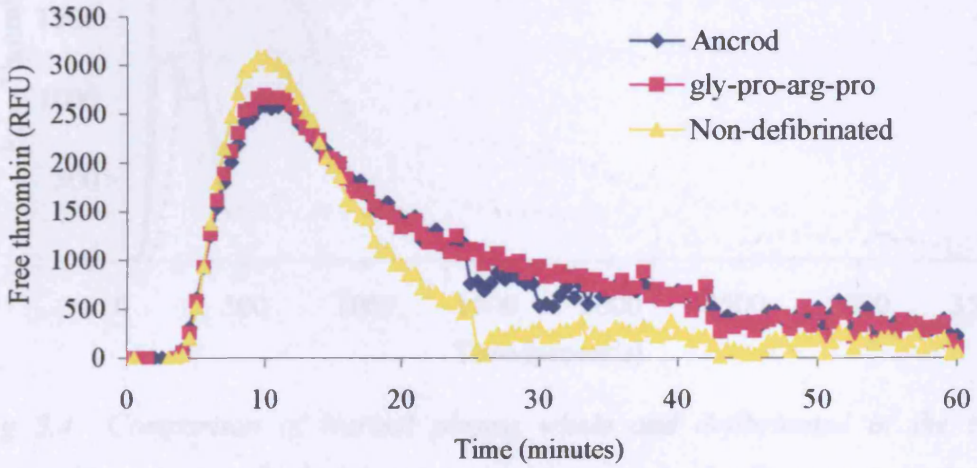


Fig 5.3. Comparison of thrombin generation by different clot inhibition treatments. Normal plasma was treated with ancrod (0.5 IU/ml) and fibrinogen removed, or gly-pro-arg-pro(4 mg/ml), n =1

5.3.3 Comparison of methods

From the curves of normal plasma (Fig 5.4) it can be seen with the fluorogenic method that the lag-time was the same for both non-defibrinated and defibrinated plasma, although the peak-height was lower and the decline in thrombin generation was more gradual. In the clotting method the lag-time was also the same, however, the peak was half that of defibrinated plasmas. The peak of the curve was also maintained for a longer duration than in defibrinated plasma. The difference in AUC was greatest with the clotting method with AUC in non-defibrinated plasma around 60% of that in defibrinated plasma, however in the flurorogenic method this was 80%. The biggest differences between methods is in the lag-time which occurs considerably later in the fluorogenic method.

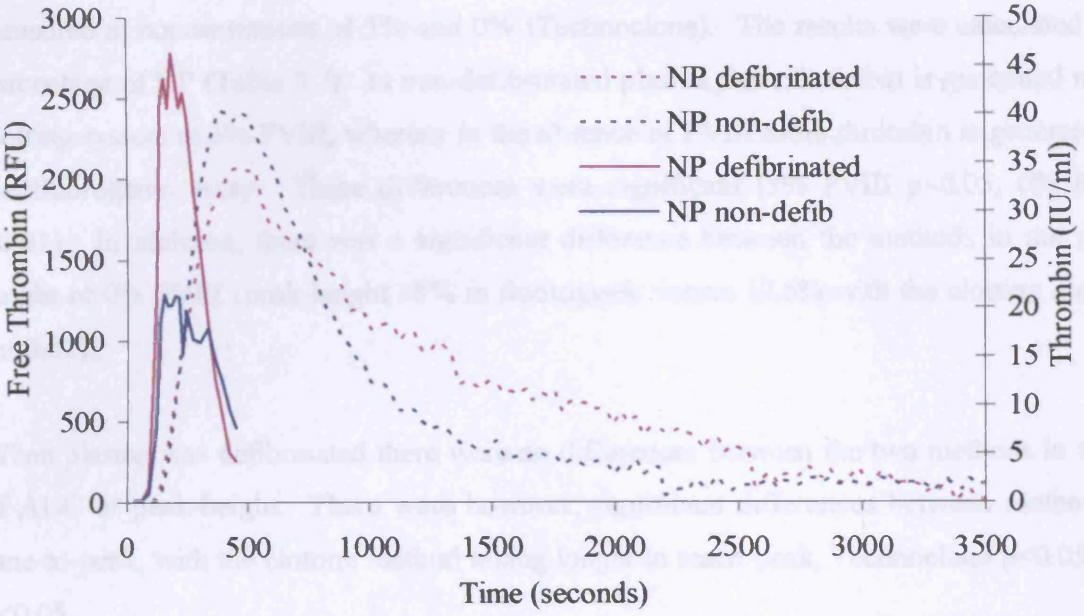


Fig 5.4 Comparison of Normal plasma whole and defibrinated in the two thrombin generation assays . Dashed lines represent assays in the fluorogenic system, solid lines represent thrombin generation in the clotting system.

Table 5.7. Comparison of thrombin generation methods at two FVIII concentrations. Parameters of 3% NP and Technoclone plasma and are given as percentage of normal plasma.

		Fluorogenic				Clotting			
		Non-defibrinated		Defibrinated		Non-defibrinated		Defibrinated	
		3%	0%	3%	0%	3%	0%	3%	0%
AUC	Mean	82.0	50.6	91.3	47.6	117.1	23.2	108.3	48.3
	SD	3.2	1.1	6.9	3.1	10.9	0.3	14.5	14.8
Peak	Mean	96.9	37.6	100.4	36.0	110.9	12.5	99.9	24.2
	SD	3.4	6.0	2.1	1.5	15.9	2.5	11.4	6.9
Time	Mean	134.9	296.0	113.8	247.8	206.3	487.7	186.4	390.6
	SD	11.7	44.4	4.1	23.5	42.7	82.7	15.7	26.3

To establish how the fluorogenic and clotting methods compared, TGT parameters were measured at concentrations of 3% and 0% (Technoclone). The results were calculated as a percentage of NP (Table 5.7). In non-defibrinated plasma more thrombin is generated in the clotting system at 3% FVIII, whereas in the absence of FVIII more thrombin is generated in the fluorogenic assay. These differences were significant (3% FVIII $p<0.05$, 0% FVIII <0.01). In addition, there was a significant difference between the methods in the peak-height of 0% FVIII (peak-height 38% in fluorogenic versus 12.5% with the clotting method ($p<0.01$)).

When plasma was defibrinated there were no differences between the two methods in terms of AUC or peak-height. There were however, significant differences between methods in time-to-peak, with the clotting method taking longer to reach peak, Technoclone $p<0.05$, 3% $p<0.05$.

The relative amounts of thrombin generated in defibrinated plasma were the same for both methods. There were differences though with non-defibrinated plasma, and this is likely to be due to the technical differences in thrombin measurement. In the fluorogenic system, the clot was not disturbed once it had formed and thrombin that was attached to the fibrin could still be detected. In contrast in the clotting system the clot must have been disturbed, in order for the subsample to be obtained and although plasma was removed from the clot some thrombin would have remained attached to the fibrin network.

5.4 Dose-response

A dose-response was carried out to further characterise the fluorogenic method over a range of FVIII concentrations. The dose-response was measured with different concentrations of *Replenate* in Technoclone FVIII deficient plasma, for both high (5nM) and low FIXa (0.08nM) trigger concentrations which are comparable to final concentrations in the clotting method (Fig 5.5, Table 5.8). The TGT parameters were then compared to NP.

High FIXa (5nM)

At the high FIXa concentration, similar results were observed to the clotting method i.e. very little change in AUC (92.1– 83.0 % of NP) with FVIII from 1 IU/ml to 0.008 IU/ml respectively and for peak-height (96.7-86.4 % of NP). As in the clotting assay there were more differences in time-to-peak, which varied by 97.5-133.3 % of NP. The deficient plasma (Technoclone) alone generated a considerable amount of thrombin, AUC 59.4±17.3 % of NP, this was a greater proportion than in the clotting assay despite the same concentration of FIXa.

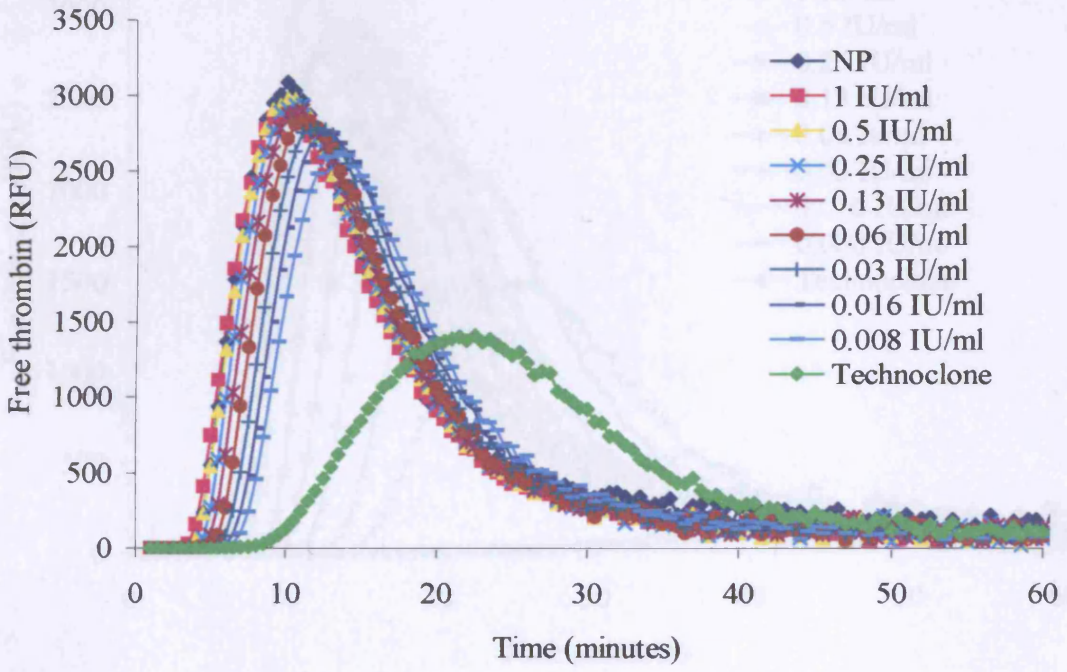


Fig 5.5. Dose-response with Replenate and a high FIXa trigger concentration. Thrombin generation was measured from 0.008-1 IU/ml Replenate in Technoclone FVIII deficient plasm, FIXa 5nM.

Low FIXa (0.08nM)

With the low FIXa concentration (0.08nM) to trigger thrombin generation (Fig 5.6, Table 5.8) there was a greater range of response to FVIII in the AUC (103±7.8-62.7±0.9%) and peak height (113.8±5.2-55.4±7.1%). There was also a reduced amount of thrombin generated in the deficient plasma in which AUC was 6.2±1.4% of that in normal plasma. There was also longer time to reach peak-height 96.0-202.9 % of NP. These results mirror those with the clotting assay.

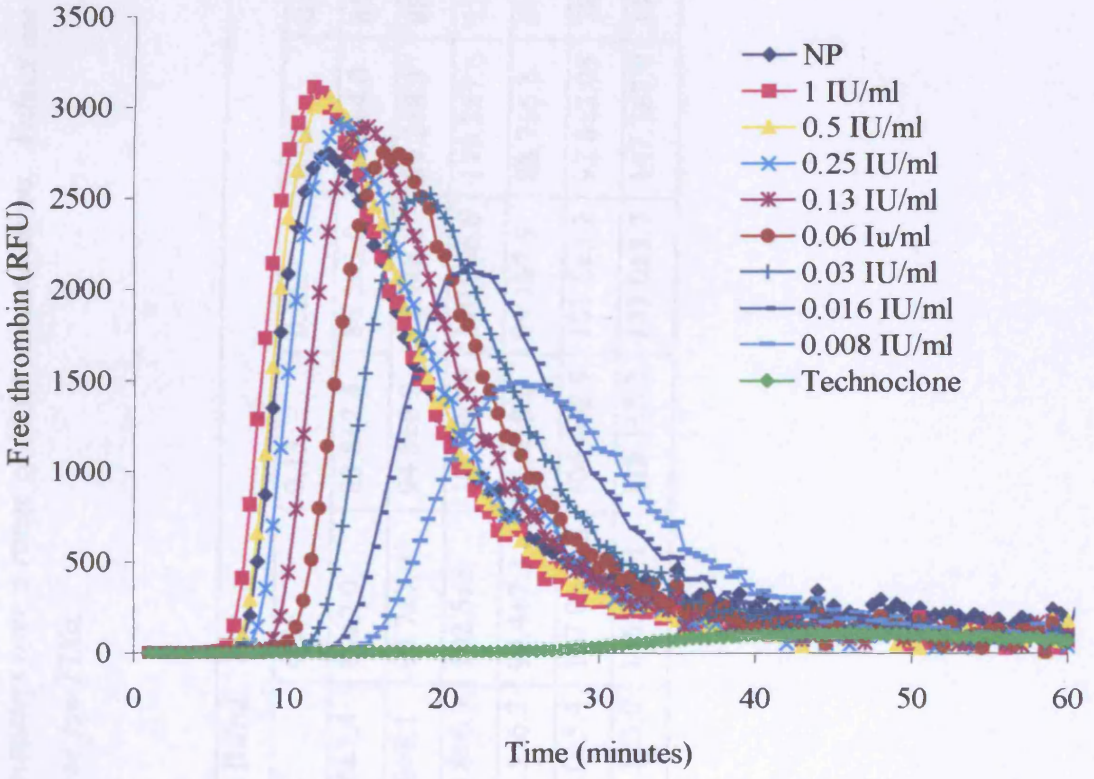


Fig 5.6. Dose-response with Replenate using 0.08nM FIXa to trigger thrombin generation. Thrombin generation was measured from 0.008-1 IU/ml FVIII in Technoclone FVIII deficient plasm, FIXa 5nM.

Table 5.8 Thrombin generation parameters over a range of FVIII concentrations. Values are expressed as a percentage of normal plasmas following activation with either high or low FIXa.

		FVIII concentration IU/ml								
		1	0.5	0.25	0.13	0.063	0.03	0.016	0.008	0
High IXa	AUC	92.1±3.7	90.6±3.4	89±2.0	88.8±2.4	86.5±3.0	84.0±4.0	85.0±3.6	83.0±4.4	59.4±17.3
	Peak	96.7±7.5	96.0±8.1	95.7±7.9	94.8±6.9	92.9±8.6	89.2±8.3	88.6±6.6	86.4±8.6	47.9±8.5
	Time	97.5±6.6	100.8±6.3	102.5±5	105.0±5.0	110.0±6.6	119.2±7.6	126.7±3.8	133.3±9.5	217.5±4.3
Low IXa	AUC	103.0±7.8	101.5±6.3	99.4±7.3	96.9±8.6	95.1±7.5	88.7±5.5	81.1±1.8	62.7±0.94	6.2±1.44
	Peak	113.8±5.2	112.0±5.4	107.0±10.3	106.0±2.9	101.5±3.2	92.8±3.05	78.3±4.7	55.4±7.1	4.33±0.7
	Time	96.0±2.0	101.3±5.0	106.8±3.1	118.1±5.3	133.0±3.7	147.7±7.0	176.0±8.1	202.9±12.1	378.4±8.8

As a large amount of thrombin was generated in commercial FVIII deficient plasma , TGT was repeated with severe haemophilic plasma (HP1 91hours) which was found to give the lowest levels of thrombin generation with the clotting method. However, HP1 91hours still generated a large amount of thrombin (Fig 5.7), AUC 46.3 % of normal plasma although this was less than the amount of thrombin generated by Technoclone.

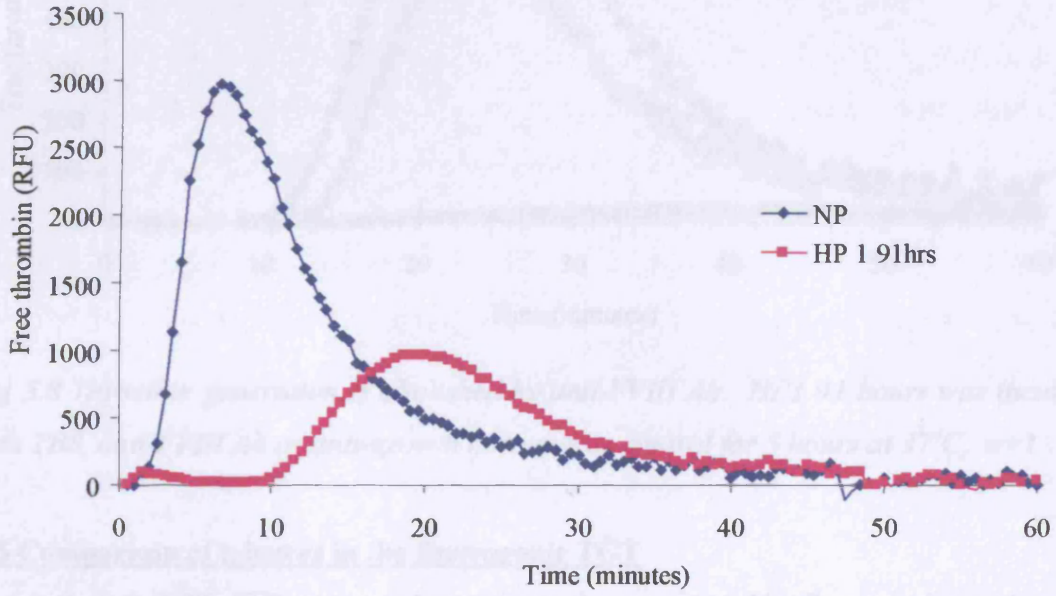


Fig 5.7 Thrombin generation in fluorogenic assay by severe haemophilic plasma.

To ensure that the large amount of thrombin generated by FVIII deficient plasmas was due to residual FVIII, as in the clotting system, a haemophilic plasma was incubated with an anti-FVIII antibody. 90µl severe haemophilic plasma (HP1 91 hours) was incubated with 10µl rabbit anti-FVIII Ab (NIBSC, 01/460) or with 10µl rabbit anti-human growth hormone serum (diluted 1 in 1000) for 5 hours at 37°C.

This treatment of the plasma abolished thrombin generation (Fig 5.8) in the haemophilic plasma treated with the anti-FVIII Ab (AUC 922 RFU.min) as opposed to the control treated plasma (10351 RFU.min).

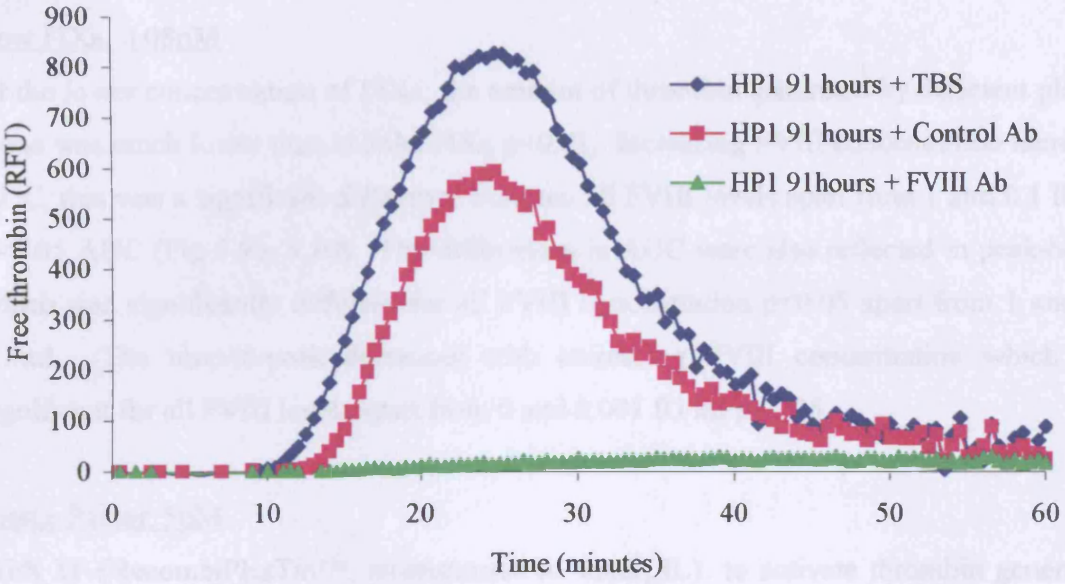


Fig 5.8 Thrombin generation is abolished by anti-FVIII Ab. HP1 91 hours was incubated with TBS, anti-FVIII Ab or anti-growth hormone as control for 5 hours at 37°C, n=1

5.5 Comparison of triggers in the fluorogenic TGT

In the clotting TGT, FIXa was used to activate the reaction. The fluorogenic test has been adapted to use similar dilutions and similar final concentrations of reagents. TF is however the most widely used trigger in TGTs and it has been suggested that a concentration of 5pM TF is akin to the *in vivo* situation (Lawson *et al*, 1994;Cawthern *et al*, 1998). In these sets of experiments a range of FVIII concentrations from 0-1 IU/ml was made by the addition of *Replenate* to a commercial FVIII deficient plasma (Technoclone). The same concentration of PL, Ca^{2+} and substrate were used, whilst variations were made with the trigger.

High FIXa. 5nM

Using the high FIXa concentration as a trigger the AUC increases (Fig 5.9a, 5.10) as FVIII concentration increases. This was however only a small increase and the AUC was only significantly lower than 1 IU/ml for deficient plasma alone, $p<0.05$ The similarity in AUC at all FVIII levels was reflected in the peak-height which plateaus at 0.01 IU/ml. The time-

to-peak decreases with increasing FVIII concentration, although there was no difference between 1 and 0.1 IU/ml and 0.1 and 0.001 IU/ml.

Low FIXa, 0.08nM

At the lower concentration of FIXa, the amount of thrombin generated by deficient plasma alone was much lower than at 5nM FIXa $p<0.01$. Increasing FVIII concentration increases AUC, this was a significant difference between all FVIII levels apart from 1 and 0.1 IU/ml $p<0.05$ AUC (Fig 5.9b, 5.10). The differences in AUC were also reflected in peak-height which was significantly different for all FVIII concentration $p<0.05$ apart from 1 and 0.1 IU/ml. The time-to-peak decreases with increasing FVIII concentration which was significant for all FVIII levels apart from 0 and 0.001 IU/ml $p<0.05$.

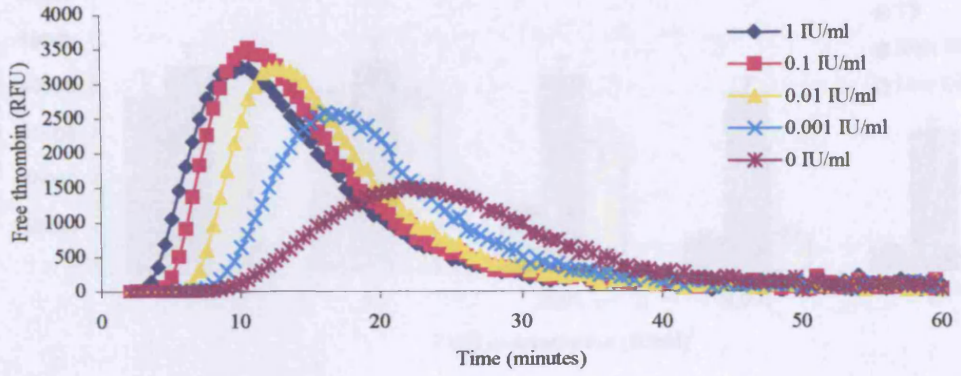
Tissue Factor, 5pM

With TF (RecombiPlasTin™, reconstituted in water, IL) to activate thrombin generation there was no difference in AUC or peak-height between 1 and 0.1 IU/ml, and 0.001 and 0 IU/ml. The differences between the other FVIII concentrations however were significant $p<0.01$. Time-to-peak was significantly different between all FVIII concentrations $p<0.05$.

Overall there was no difference in peak-height or amount of thrombin generated at 1 and 0.1 IU/ml FVIII between the different triggers. In terms of the AUC there was no difference between 0.08nM FIXa and 5pM FIXa apart from at 0.01 IU/ml, at this concentration the low FIXa trigger generates significantly more thrombin than TF ($p<0.05$). There were significant differences in time-to-peak between all methods.

To summarise, the amount of thrombin generated at low FVIII concentrations is affected by the type and concentration of trigger used. The low FIXa concentration gave a greater differences in lag-time between the FVIII concentrates and the concomitant changes in peak height and AUC. The difference in the amount of thrombin generated at 0.01 IU/ml by the different methods is crucial, as this is the level that defines severe haemophilia. The sensitivity and reproducibility of the assay at low FVIII levels (0.001-0.1 IU/ml) was greater when using the low FIXa as the trigger. For all studies hereonin with the fluorogenic method the low FIXa concentration was used, unless otherwise stated.

A



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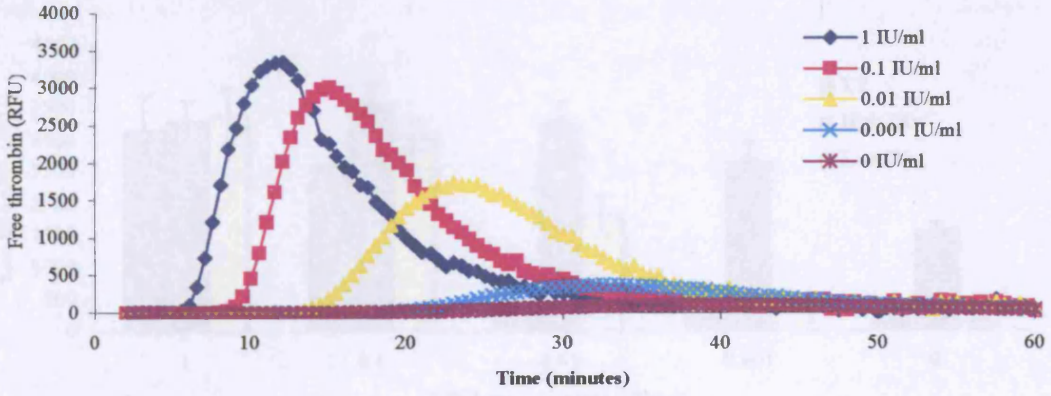

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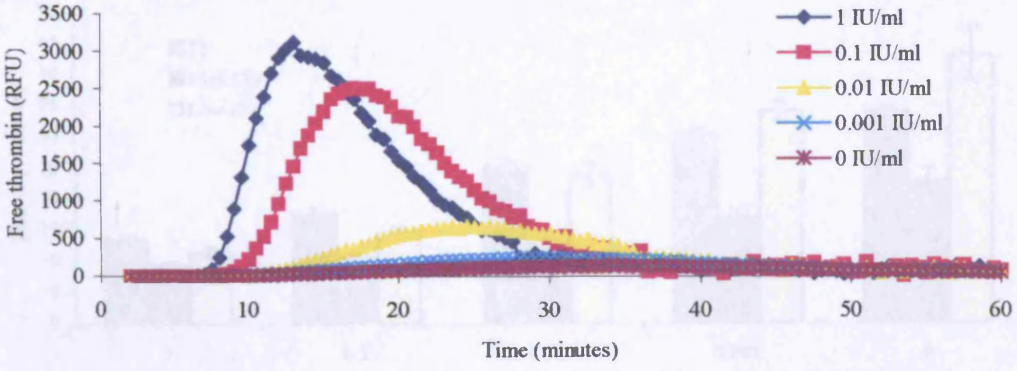
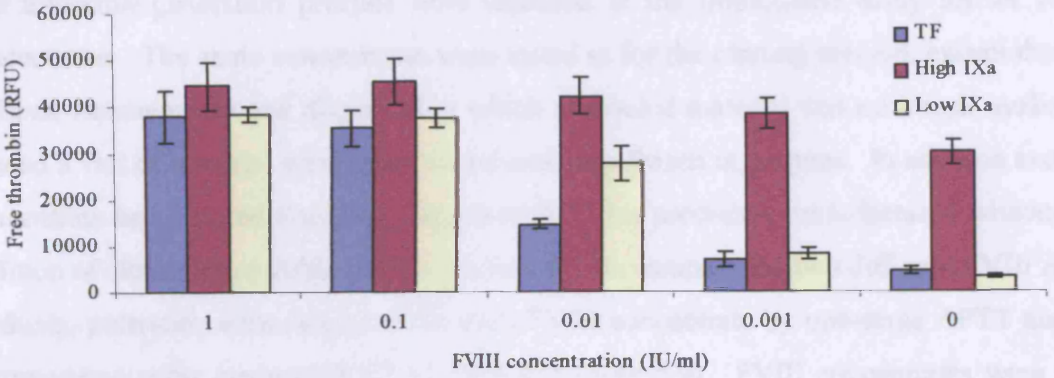


Fig 5.9 Comparisons of Triggers for thrombin generation. A- High IXa, B- Low IXa, C- TF 5pM. FVIII concentration 1-0 IU/ml.

A



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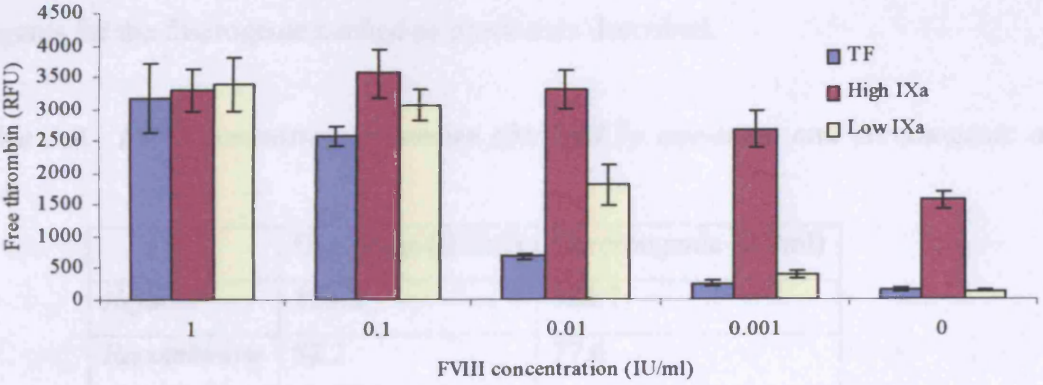

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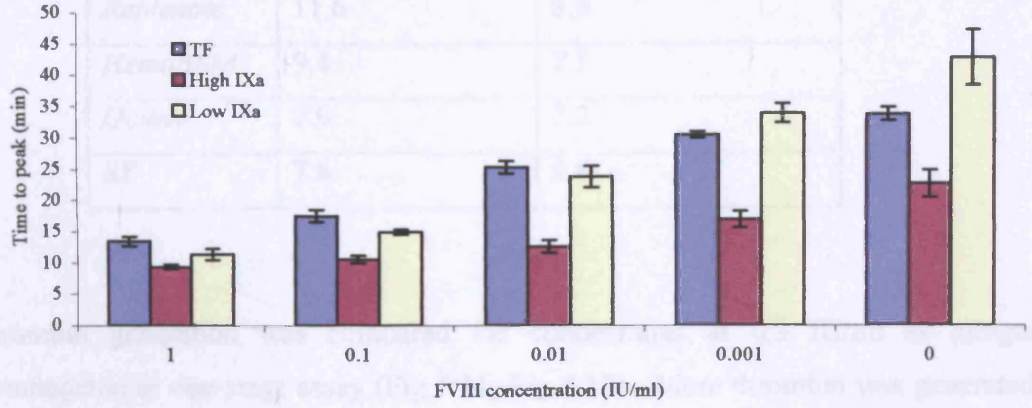


Fig 5.10 Comparison of thrombin generation parameters A- AUC, B- Peak height C- Time-to-peak.

5.6 Comparison of FVIII concentrates

The thrombin generation profiles were assessed in the fluorogenic assay for all FVIII concentrates. The same concentrates were tested as for the clotting method, except that for *Refacto*, *Recombinate* and *Kogenate* in which ampouled material was no longer avaliable, instead a vial of material was reconstituted and snap frozen in aliquots. In addition another concentrate had become available "*Kogenate-SF*" this product is manufactured without the addition of albumin to stabilise the final product. To compare the two different FVIII assay methods, potencies were measured for each FVIII concentrate by one-stage APTT and by chromogenic assay against the 6th IS (NIBSC)(Table 5.9). FVIII concentrates were then diluted in FVIII deficient plasma (Technoclone) to achieve a concentration of 0.3 IU/ml according to potency. Thrombin generation was then triggered with low FIXa and other reagents for the fluorogenic method as previously described.

Table 5.9. FVIII concentrate potencies obtained by one-stage and chromogenic assays n=3.

	One-stage (IU/ml)	Chromogenic (IU/ml)
Refacto	123.2	188.7
Recombinate	82.2	77.6
Kogenate	92.3	84.9
Kogenate-SF	24.1	19.3
Replenate	11.6	8.9
Hemofil-M	9.4	7.1
Octavi	7.9	7.2
8Y	7.8	8.8

Thrombin generation was compared for concentrates at 0.3 IU/ml as assigned by chromogenic or one-stage assay (Fig 5.11, Fig 5.12). More thrombin was generated when chromogenic potencies were used, this was also true for peak-height. These differences though were not statistically significant. In general there was a wider spread in time-to-peak with the one-stage assay compared to chromogenic. Time-to-peak was slightly longer

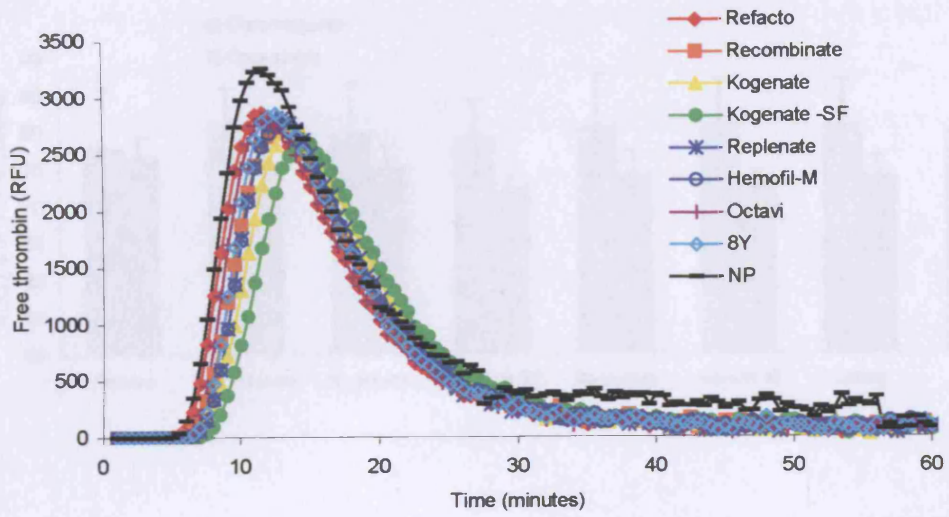
in the one-stage than the chromogenic, but this was again not significant. The one concentrate that did not generate thrombin more slowly in the one-stage assay was *Refacto* which had time-to-peak of 110% in chromogenic compared to 102.5% of NP with the one-stage potency.

With the one-stage potency, *Refacto* generated thrombin more rapidly that other concentrates, this though was not statistically significant from all other concentrates. There were statistical differences between the concentrates in time-to-peak as measured by ANOVA $p<0.001$.

In the chromogenic assay, time-to-peak is more closely clustered with the exception of *Kogenate*-SF. The variation between all concentrates was significant (ANOVA $p<0.05$), however, this could be attributed to *Kogenate*-SF as removal of this from ANOVA analysis resulted in no significant differences between the remaining concentrates $p=0.35$.

Overall there was no difference in the amount of thrombin generated by the concentrates when potencies were measured with either assay. When potencies were measured by chromogenic assay it resulted in thrombin generation profiles which were most similar, and would suggest that chromogenic assay potencies correlate more closely with thrombin potential.

A



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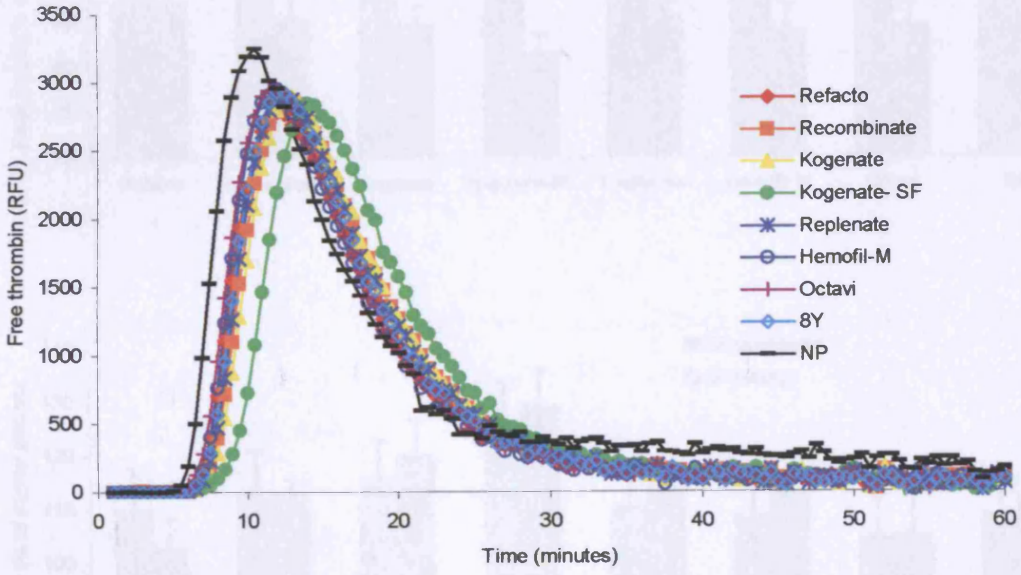
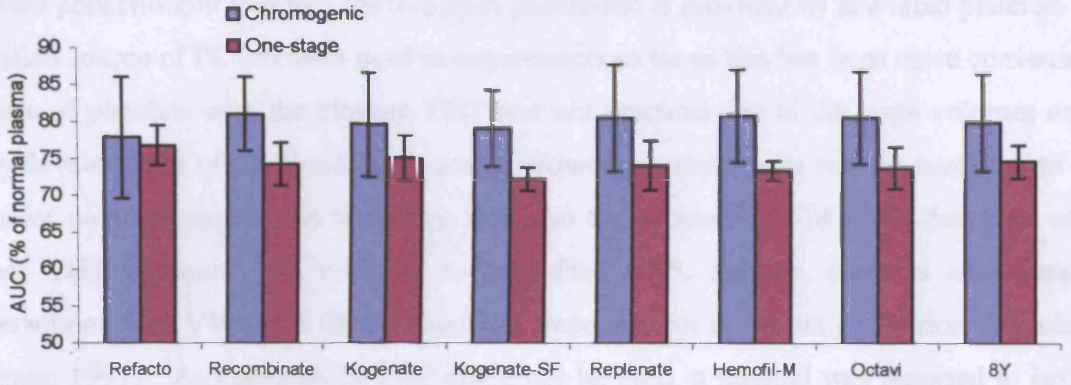


Fig 5.11 Thrombin generation by all FVIII concentrates at 0.3 IU/ml, FIXa 0.08nM. A- potency measured by one-stage assay B- potency measured by chromogenic assay n=4.

A



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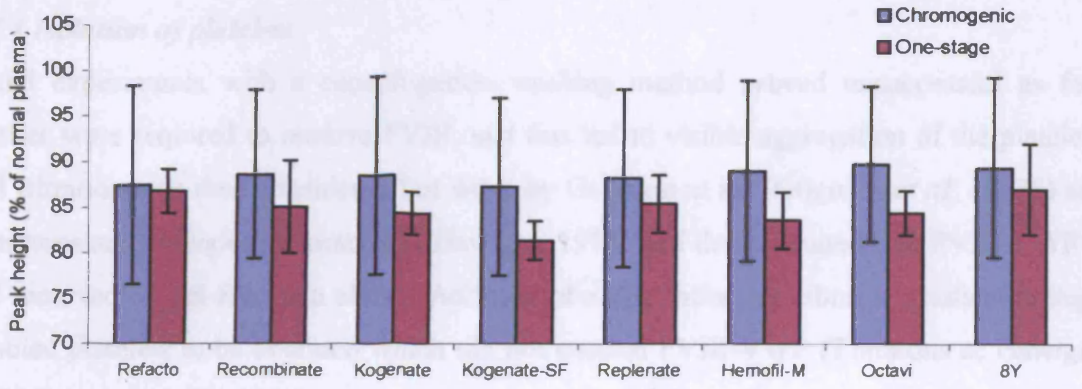

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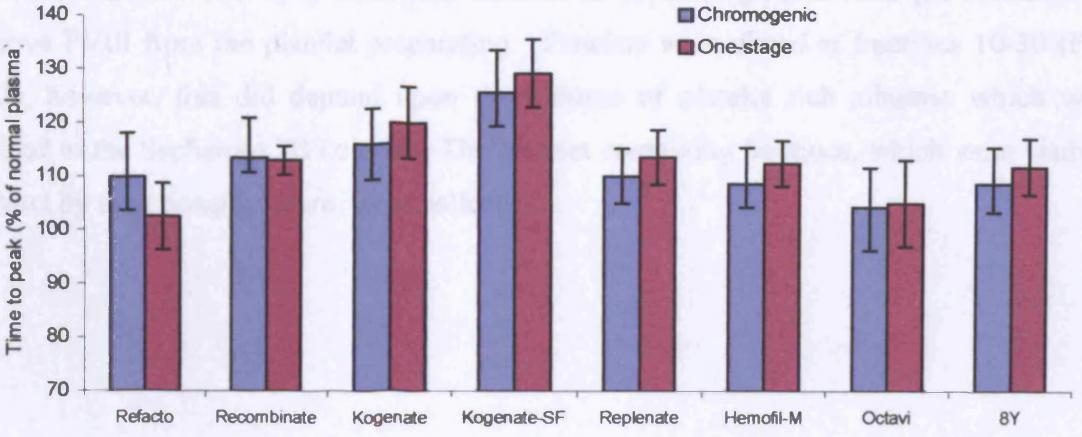


Fig 5.12 Curve parameters of concentrates in comparison with normal plasma. A-AUC B-Peak-height C-Time-to-peak, n=4.

5.7 Platelets

In vivo phospholipid required for thrombin generation is provided by activated platelets. A purified source of PL has been used in experiments so far as this has been more convenient, as use of platelets with the clotting TGT was not practical due to the large volumes used. The development of the fluorogenic assay allowed platelets to be readily used due to the smaller volumes required in the assay, and also the developing clot is not disturbed as in other TGT methods. In addition to providing a PL surface, platelets also support interactions with VWF and fibrin which are important for thrombin generation (Béguin & Kumar, 1997). As haemophilic PRP could not be used, a method was required to isolate platelets from normal donor PRP and remove FVIII while avoiding platelet activation.

5.7.1 Isolation of platelets

Initial experiments with a centrifugation washing method proved unsuccessful as four washes were required to remove FVIII, and this led to visible aggregation of the platelets. Gel filtration was then considered but work by Grignani et al (Grignani *et al*, 1977b) and Timmons and Hawgier (Timmons & Hawiger, 1978) had demonstrated that FVIII-VWF is not removed by gel-filtration alone. Addition of a discontinuous albumin gradient though, enabled platelets to be obtained which did not contain FVIII-VWF (Timmons & Hawiger, 1978;Grignani *et al*, 1977a).

Platelets were isolated by a combined method of albumin gradient and gel-filtration to remove FVIII from the platelet preparation. Platelets were eluted at fractions 10-30 (Fig 5.13), however, this did depend upon the volume of platelet rich albumin which was applied to the Sepharose 2B column. The platelet containing fractions, which were visibly distinct by their opaque nature, were collected.

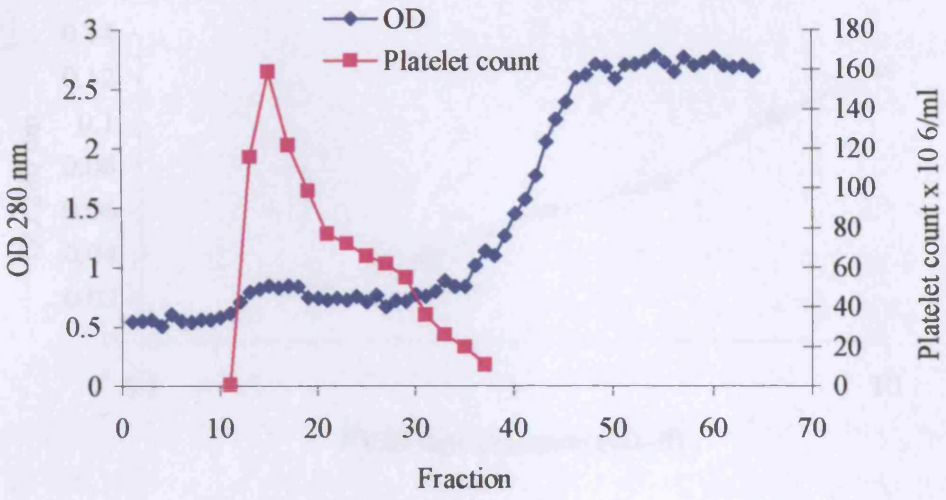
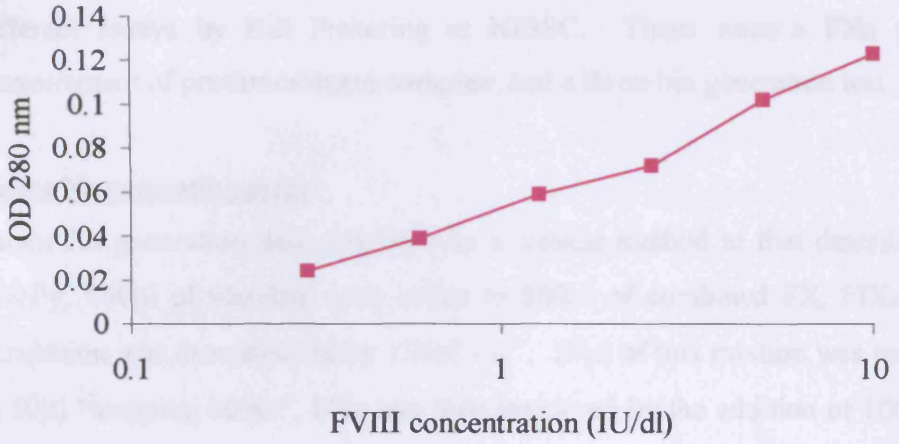
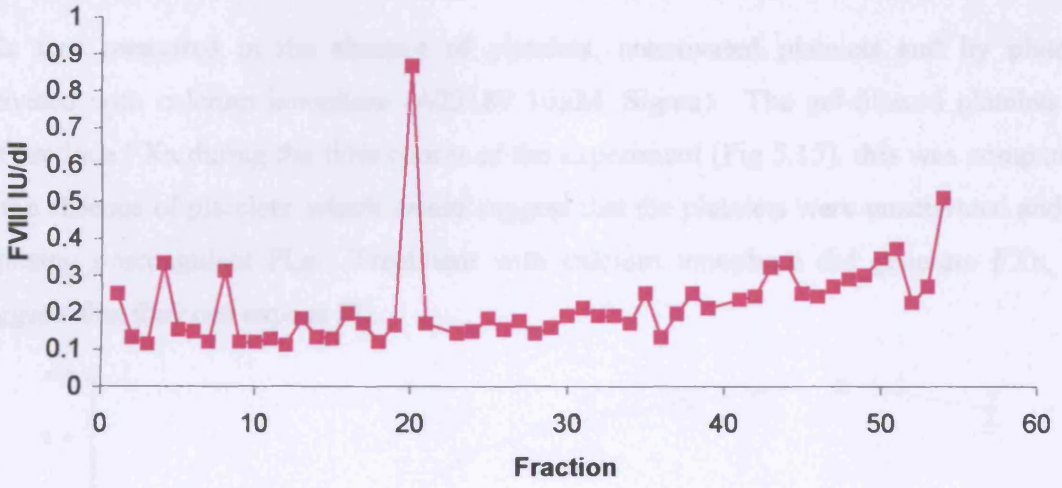


Fig 5.13 A typical elution profileof platelets from a sepharose 2B column.

All fractions eluted were measured for protein by OD at 280nm. Platelets are eluted before most of the proteins (Fig 5.13). To make sure that FVIII was not eluted in the platelet containing fractions a FVIII ELISA was carried out. In the example presented here fraction 1-54 were analysed (Fig 5.14). All fractions contained FVIII less than 1 IU/dl, and all but one of the platelet fractions contained less than 0.3 IU/dl. This fraction is most probably an aberrant reading as fractions either side were much lower.



A



B

Fig 5.14 FVIII ELISA of gel filtration fractions. A- Standard curve B- FVIII content of fractions. Results from one experiment.

The pooled platelet preparation was also tested and found to contain 0.02 IU/dl FVIII. This method of isolating platelets was therefore found to contain minimal FVIII.

To ensure that the platelets were not activated by the process they were tested in three different assays by Bill Pickering at NIBSC. These were a FXa generating assay, measurement of prothrombinase complex, and a thrombin generation test.

Factor Xa generating assay

Factor Xa generation was measured by a similar method to that described in section 2.4 Briefly, 160µl of platelets were added to 800µl of combined FX, FIXa and FVIII, FXa generation was then initiated by 160µl Ca^{2+}. 25µl of this mixture was removed and added to 50µl "stopping buffer", FXa was then measured by the addition of 100µl 3mM S-2756, this reaction was terminated at 3 minutes with 50% acetic acid. The amount of FXa was determined from a standard curve.

FXa was measured in the absence of platelets, unactivated platelets and by platelets activated with calcium ionophore (A23187 10µM, Sigma). The gel-filtered platelets did not produce FXa during the time course of the experiment (Fig 5.15), this was comparable to the absence of platelets, which would suggest that the platelets were unactivated and not exposing procoagulant PLs. Treatment with calcium ionophore did generate FXa, this suggests that they can expose PL.

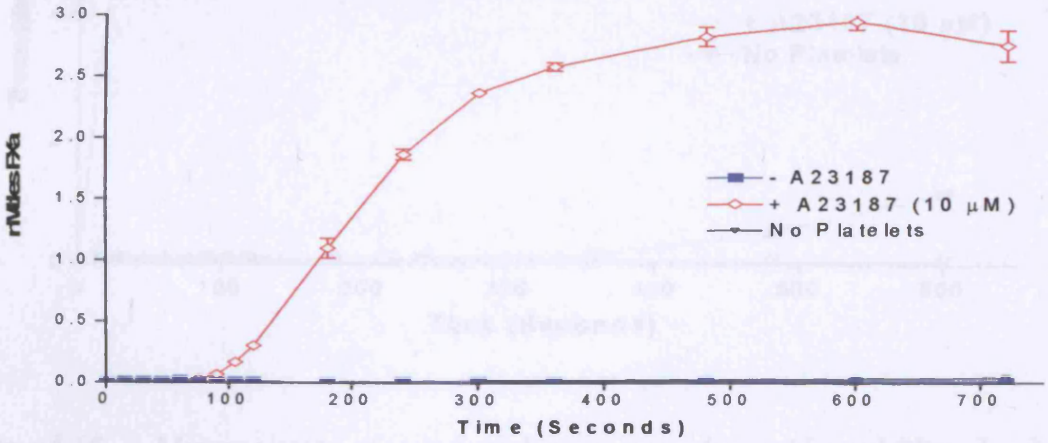


Fig 5.15 FXa generation by platelets. FXa generation: Effect of calcium ionophore (A23187) on gel-filtered platelets at a concentration of 100x10^9/L

Prothrombinase complex

The ability of the platelets to partake in the prothrombinase complex was measured by incubating 158µl of platelets for 3 minutes with 5µl Ca^{2+}, 25µl FXa (3mM), and FVa (6mM, Hematologic Technologies Inc). Prothrombin 60µl was added to reach a final concentration of 4µM. 5µl of reaction mixture was removed at timed intervals into 200µl "stopping buffer". The amount of thrombin generated was determined by addition of 50µl S-2238 (Chromogenix) for 3 minutes, subsequently colour reaction was terminated with 50µl 50% acetic acid.

Gel-filtered platelets only started to produce thrombin after 350 seconds (Fig 5.16). Whereas in ionophore-treated platelets maximal thrombin was produced within 75 seconds. This demonstrates that without activation the gel-filtered platelets are much less able to provide the necessary phospholipid surface for the prothrombinase complex.

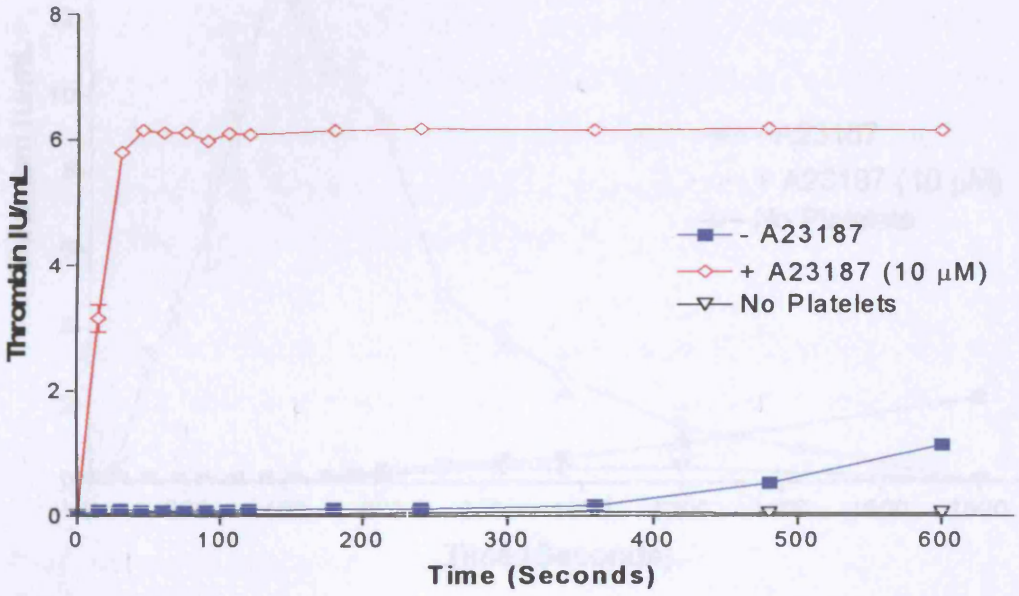


Fig 5.16 Measurement of prothrombinase complex with gel-filtered platelets. Prothrombinase activity: Effect of calcium ionophore (A23187) on gel-filtered platelets at a concentration of 100x10^9/L.

Thrombin generation

Thrombin generation in plasma was also measured in a chromogenic assay. 80µl FIXa (1.1 IU/ml) and 400µl defibrinated normal pooled plasma were mixed, 100µl of platelets were then added, thrombin generation was initiated with 50µl Ca^{2+}(90mM), 10µl subsamples were taken at intervals into 140µl stopping buffer. Thrombin was detected by adding 50µl S-2238 (1.2mM) for 3 minutes, then terminating with 80µl 50% acetic acid.

A small amount of thrombin was generated by the unactivated platelets by 30 minutes (Fig 5.17). This is in contrast to the ionophore-treated platelets which reached maximal thrombin generation at 390 seconds.

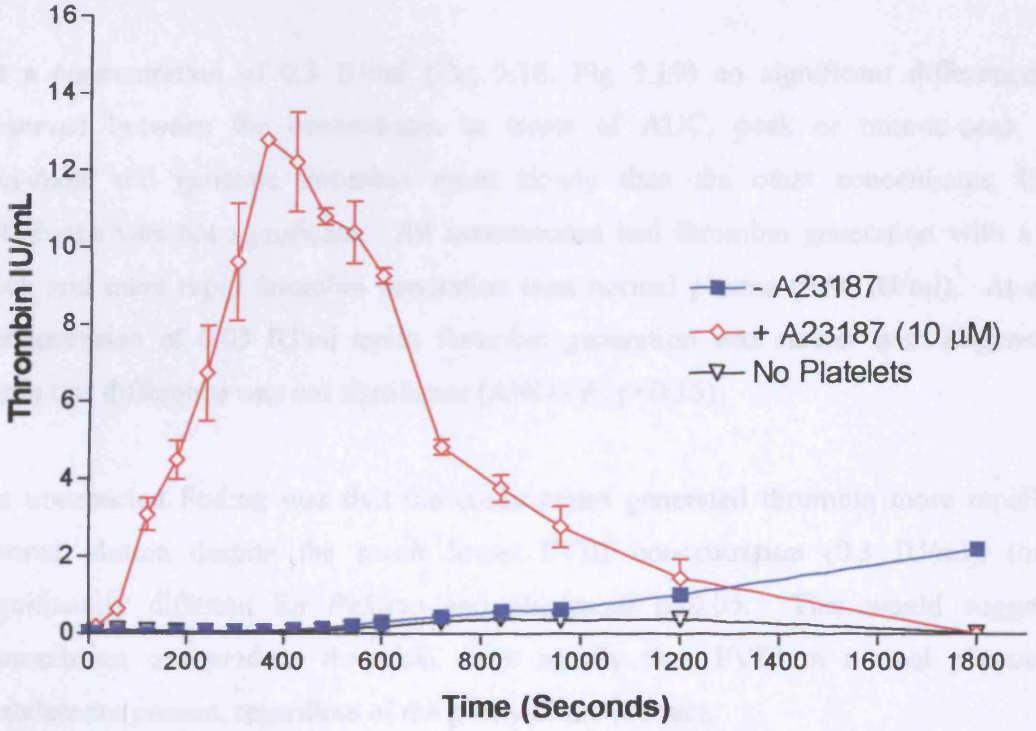


Fig 5.17 Thrombin generation in normal plasma by gel filtered platelets.

These experiments demonstrated that the platelets, following albumin gradient and gel filtration were in an unactivated state. They were considerably slower to partake in the prothrombinase complex and thrombin generation, and no FXa was generated during the

time-course of this experiment. Therefore gel-filtered platelets contained minimal FVIII and remained in an unactivated state.

5.7.2 FVIII concentrates with platelets

Four different concentrates (*Refacto*, *Kogenate*, *Replenate* and *8Y*) were compared at 0.3 and 0.03 IU/ml with unactivated platelets to establish if there was any difference between the types of concentrates in their ability to generate thrombin in the presence of platelets.

Thrombin generation was measured with 40µl HP 91hours plasma, with 40µl platelets ($200x10^6$/ml), thrombin generation was initiated by the addition of 40µl substrate mix (substrate 0.7nM, FIXa 0.23nM, Ca^{2+}21 mM).

At a concentration of 0.3 IU/ml (Fig 5.18, Fig 5.19) no significant differences were observed between the concentrates in terms of AUC, peak or time-to-peak height. *Kogenate* did generate thrombin more slowly than the other concentrates, but this difference was not significant. All concentrates had thrombin generation with a higher peak and more rapid thrombin generation than normal plasma (0.86 IU/ml). At a lower concentration of 0.03 IU/ml again thrombin generation was slower with *Kogenate*, but again this difference was not significant (ANOVA, $p=0.35$).

An unexpected finding was that the concentrates generated thrombin more rapidly than normal plasma despite the much lower FVIII concentration (0.3 IU/ml), this was significantly different for *Refacto* and *Replenate* $p<0.05$. This would suggest that concentrates can produce thrombin more rapidly than FVIII in normal plasma when platelets are present, regardless of the purity of the product.

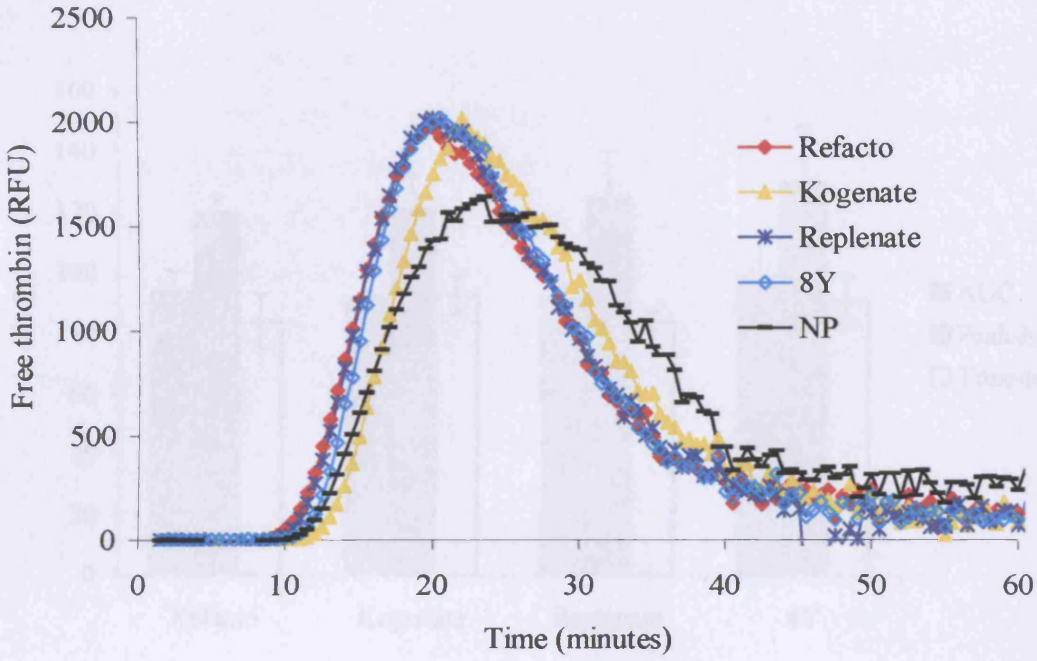
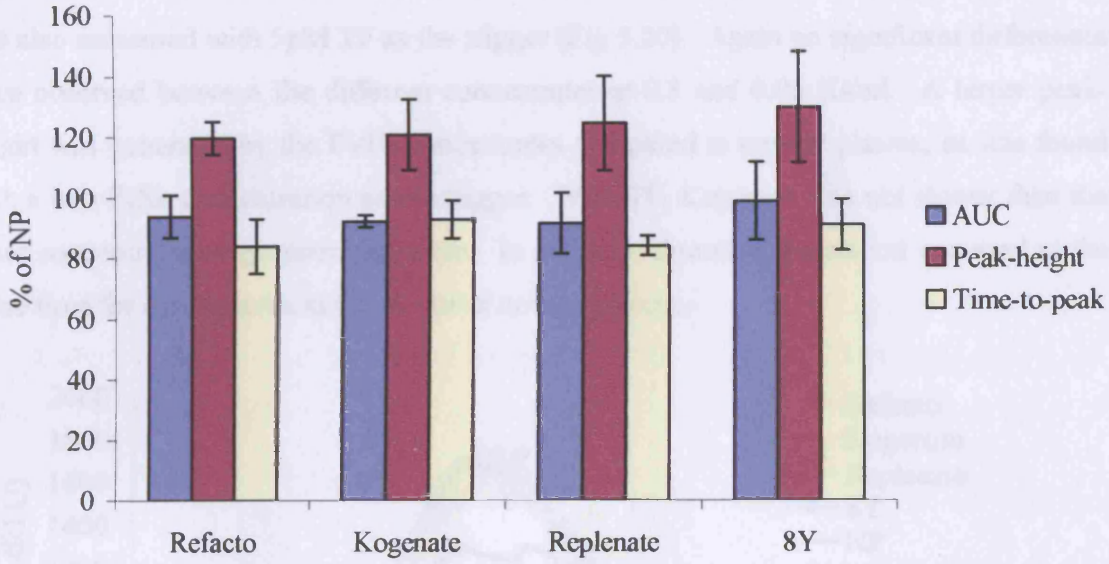


Fig 5.18. Thrombin generation by FVIII concentrates (0.3 IU/ml) triggered with low FIXa and with platelets.

A



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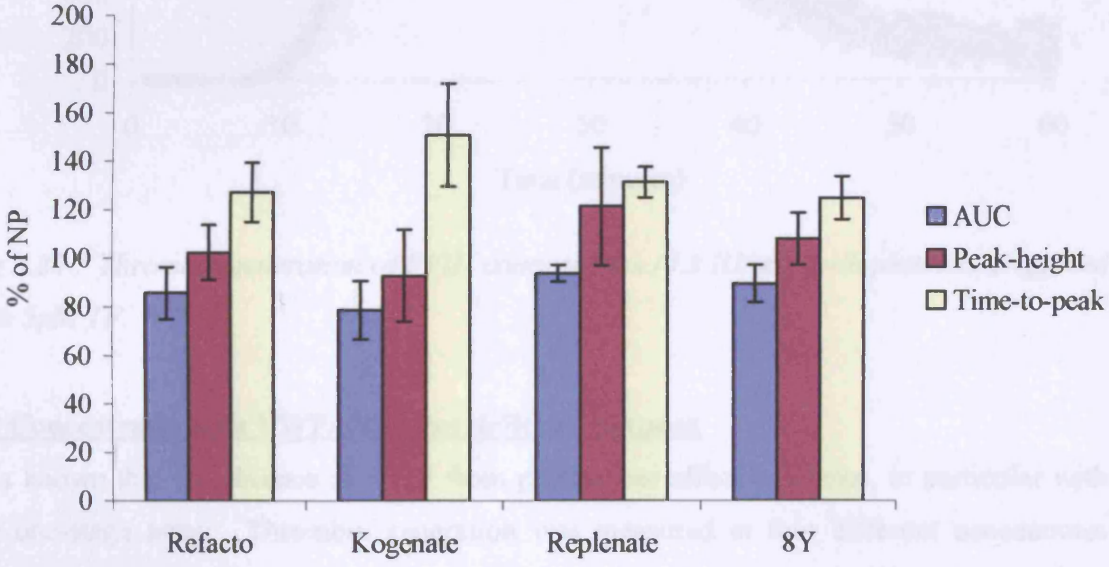


Fig 5.19 Thrombin generation parameters of FVIII concentrate with platelets A- Concentrates 0.3 IU/ml B- Concentrates 0.03 IU/ml.

Just to make sure that there was no difference between concentrates, thrombin generation was also measured with 5pM TF as the trigger (Fig 5.20). Again no significant differences were observed between the different concentrates at 0.3 and 0.03 IU/ml. A larger peak-height was generated by the FVIII concentrates compared to normal plasma, as was found with a low FIXa concentration as the trigger. With TF, *Kogenate* was not slower than the other concentrates to generate thrombin. In addition, thrombin generation occurred at the same time for concentrates at 0.3 IU/ml as normal plasma.

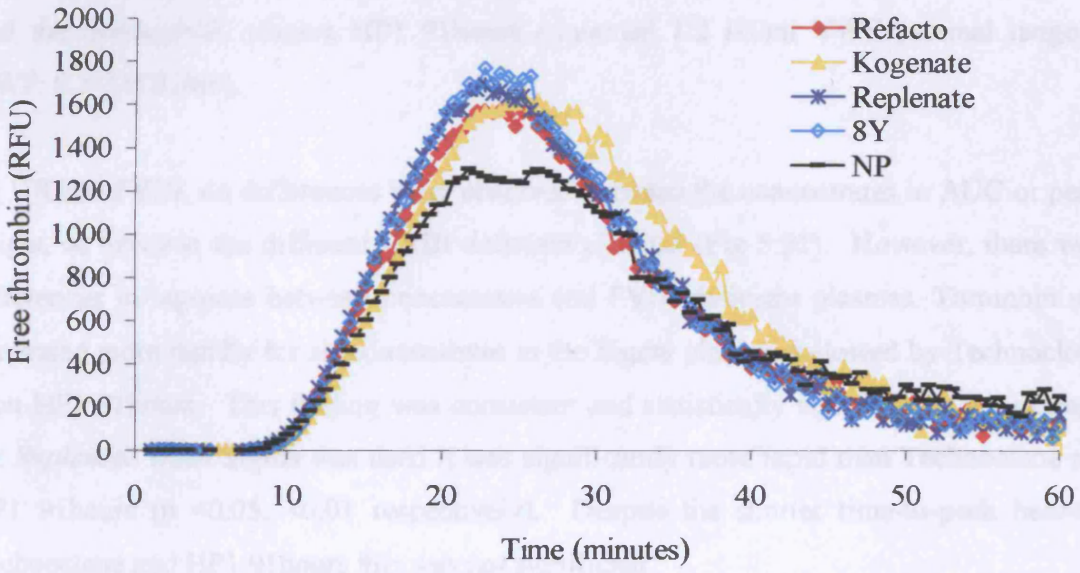


Fig 5.20 . Thrombin generation of FVIII concentrates (0.3 IU/ml), with platelets, triggered with 5pM TF.

5.8 Concentrates with VWF/ different deficient plasmas

It is known that the absence of VWF from plasma can affect potencies, in particular with the one-stage assay. Thrombin generation was measured in four different concentrates (*Refacto*, *Kogenate*, *Replenate*, *8Y*). Of these concentrates tested only *8Y*, an intermediate purity product contains a large amount of VWF, which contains VWF:Ag:FVIII:c 2.9:1 whereas *Replenate* contains 0.013:1 (Raut *et al*, 1999), the recombinant products of course do not contain any VWF.

Thrombin generation was measured for the four concentrates in three different FVIII deficient plasmas. The plasma from Sigma was specifically chosen as VWF is removed during the manufacture. The other commercial plasma, Technoclone, contained normal VWF levels. They were both compared to severe haemophilic plasma which also contained normal VWF levels. Thrombin generation was measured by incubating 40µl plasma/concentrate with 40µl PL and 40µl substrate/Ca^{2+}/FIXa mixture.

The FVIII deficient plasmas were assessed for VWF content by Lynne Weller (NIBSC). The plasma manufactured by Sigma contained no VWF, Technoclone contained 0.62 IU/ml and the haemophilic plasma HP1 91hours contained 1.2 IU/ml VWF (normal range of VWF: 0.5-2.0 IU/ml).

At 1 IU/ml FVIII, no differences were observed between the concentrates in AUC or peak-height, or between the different FVIII deficient plasmas (Fig 5.21). However, there were differences in lag-time between concentrates and FVIII deficient plasmas. Thrombin was generated more rapidly for all concentrates in the Sigma plasma, followed by Technoclone, then HP1 91hours. This finding was consistent and statistically significant. For example for *Replenate* when Sigma was used it was significantly more rapid than Technoclone and HP1 91hours ($p < 0.05$, < 0.01 respectively). Despite the shorter time-to-peak between Technoclone and HP1 91hours this was not significant.

The only difference between concentrates was observed for *Refacto*, *Kogenate* and *8Y* where the two high purity products generated thrombin significantly more rapidly than *8Y* ($p < 0.01$, < 0.05, respectively) in the Sigma plasma. These differences were not observed in the other FVIII deficient plasmas. In HP1 91hours thrombin was generated at approximately the same time for all concentrates. These results suggest that the absence of VWF causes thrombin to be generated more rapidly.

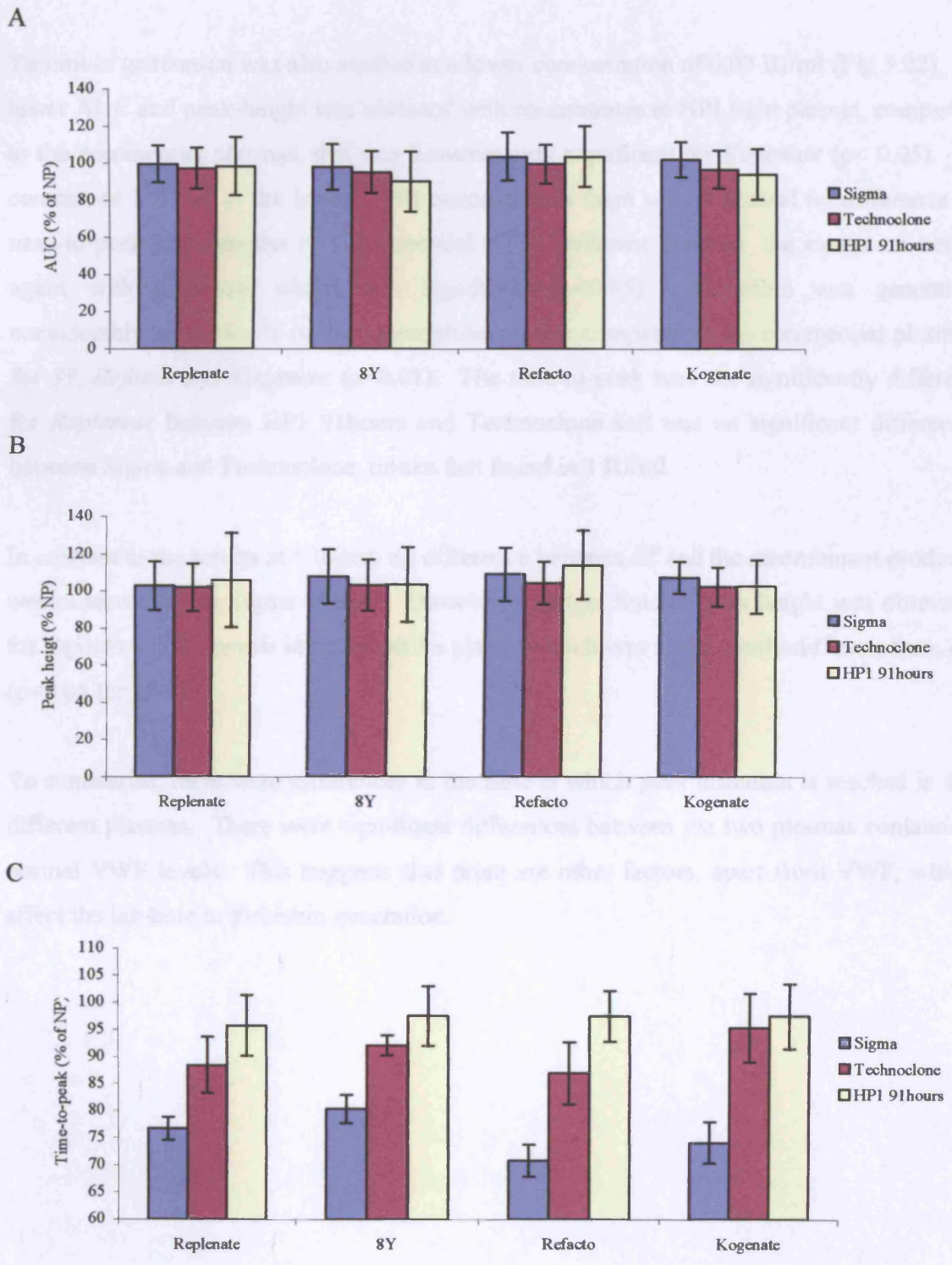


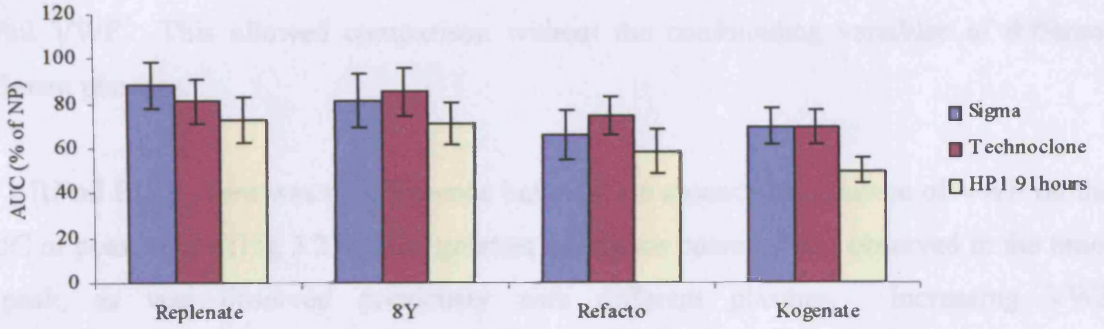
Fig 5.21. Thrombin generation parameters for FVIII concentrates (1 IU/ml) in different FVIII deficient plasmas. A-AUC B-Peak-height C-Time-to-peak

Thrombin generation was also studied at a lower concentration of 0.03 IU/ml (Fig 5.22). A lower AUC and peak-height was obtained with concentrates in HP1 91hr plasma, compared to the commercial plasmas, this was however only significant for *Kogenate* ($p< 0.05$). In contrast to 1 IU/ml at the lower FVIII concentration there was in general no difference in time-to-peak between the two commercial FVIII deficient plasmas, the exception being again with *Kogenate* which was significant ($p<0.05$). Thrombin was generated considerably more slowly in the haemophilic plasma compared to the commercial plasmas for *8Y*, *Refacto* and *Kogenate* ($p<0.01$). The time-to-peak was not significantly different for *Replenate* between HP1 91hours and Technoclone and was no significant difference between Sigma and Technoclone, unlike that found in 1 IU/ml.

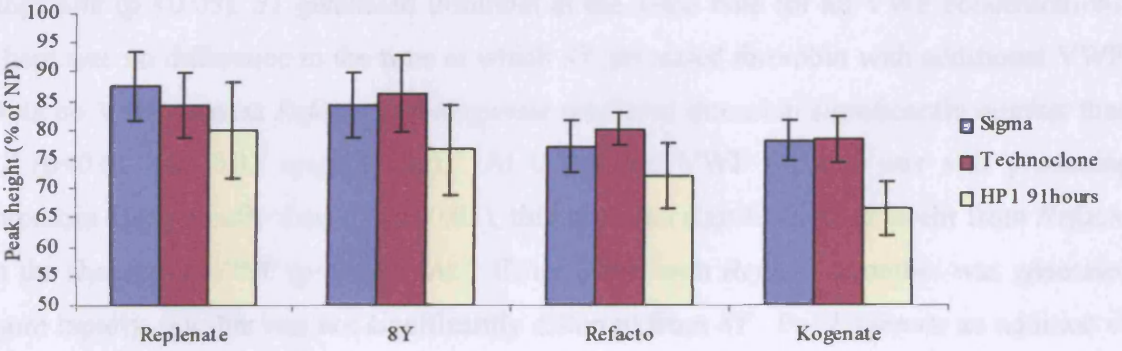
In contrast to the results at 1 IU/ml, no difference between *8Y* and the recombinant products was observed in the Sigma plasma. However, a longer time-to-peak height was observed for *Refacto* and *Kogenate* in haemophilic plasma which was significantly different from *8Y* ($p<0.05$ for both).

To summarise, there were differences in the time at which peak thrombin is reached in the different plasmas. There were significant differences between the two plasmas containing normal VWF levels. This suggests that there are other factors, apart from VWF, which affect the lag-time in thrombin generation.

A



B



C

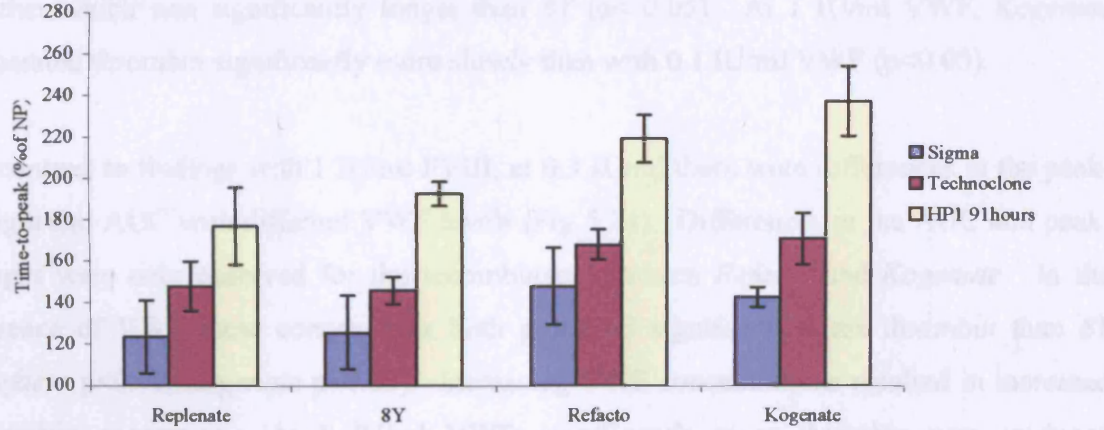


Fig 5.22. Thrombin generation by FVIII concentrates (0.03 IU/ml) in different FVIII deficient plasmas A- AUC, B Peak-height C-Time-to-peak

To assess the direct influence of VWF on thrombin generation, purified VWF (1st IS 00/514) was added to Sigma (which did not contain VWF) to concentrations of 0.1 and 1 IU/ml VWF. This allowed comparison without the confounding variables of different deficient plasmas.

At 1 IU/ml FVIII there was no difference between the absence or presence of VWF on the AUC or peak height (Fig 5.23). The greatest difference however was observed in the time-to-peak, as was observed previously with different plasmas. Increasing VWF concentration, increased the time-to-peak for *Replenate*, *Refacto* and *Kogenate*, although the difference between 0 and 1 IU/ml VWF was only significant for *Refacto* ($p < 0.05$) and *Kogenate* ($p < 0.05$). *8Y* generated thrombin at the same time for all VWF concentrations. There was no difference in the time at which *8Y* generated thrombin with additional VWF. With no VWF present *Refacto* and *Kogenate* produced thrombin significantly quicker than *8Y* ($p<0.01$ and 0.05 respectively). At 0.1 IU/ml VWF *Refacto* was still producing thrombin more rapidly than *8Y* ($p<0.05$), this was also significantly different from *Refacto* in the absence of VWF ($p<0.05$). At 1 IU/ml VWF with *Refacto* thrombin was generated more rapidly, but this was not significantly different from *8Y*. For *Kogenate* an addition of 0.1 IU/ml VWF to the FVIII deficient plasma increased the time-to-peak, to that of *8Y*, a further increase in the VWF concentration to 1 IU/ml prolonged the time-to-peak height further which was significantly longer than *8Y* ($p< 0.05$). At 1 IU/ml VWF, *Kogenate* generated thrombin significantly more slowly than with 0.1 IU/ml VWF ($p<0.05$).

In contrast to findings with 1 IU/ml FVIII, at 0.3 IU/ml there were differences in the peak-height and AUC with different VWF levels (Fig 5.24). Differences in the AUC and peak-height were only observed for the recombinant products *Refacto* and *Kogenate*. In the absence of VWF these concentrates both produced significantly less thrombin than *8Y* (*Refacto* $p<0.01$, *Kogenate* $p<0.05$). Increasing VWF concentrations resulted in increased thrombin generation. At 1 IU/ml VWF, significantly more thrombin was produced compared to 0 IU/ml for *Refacto* ($p<0.05$) and *Kogenate* ($p<0.05$). These findings were also mirrored in the findings with the peak-height. An unexpected finding was that at the lower FVIII concentration time-to-peak was unaffected by increasing VWF levels.

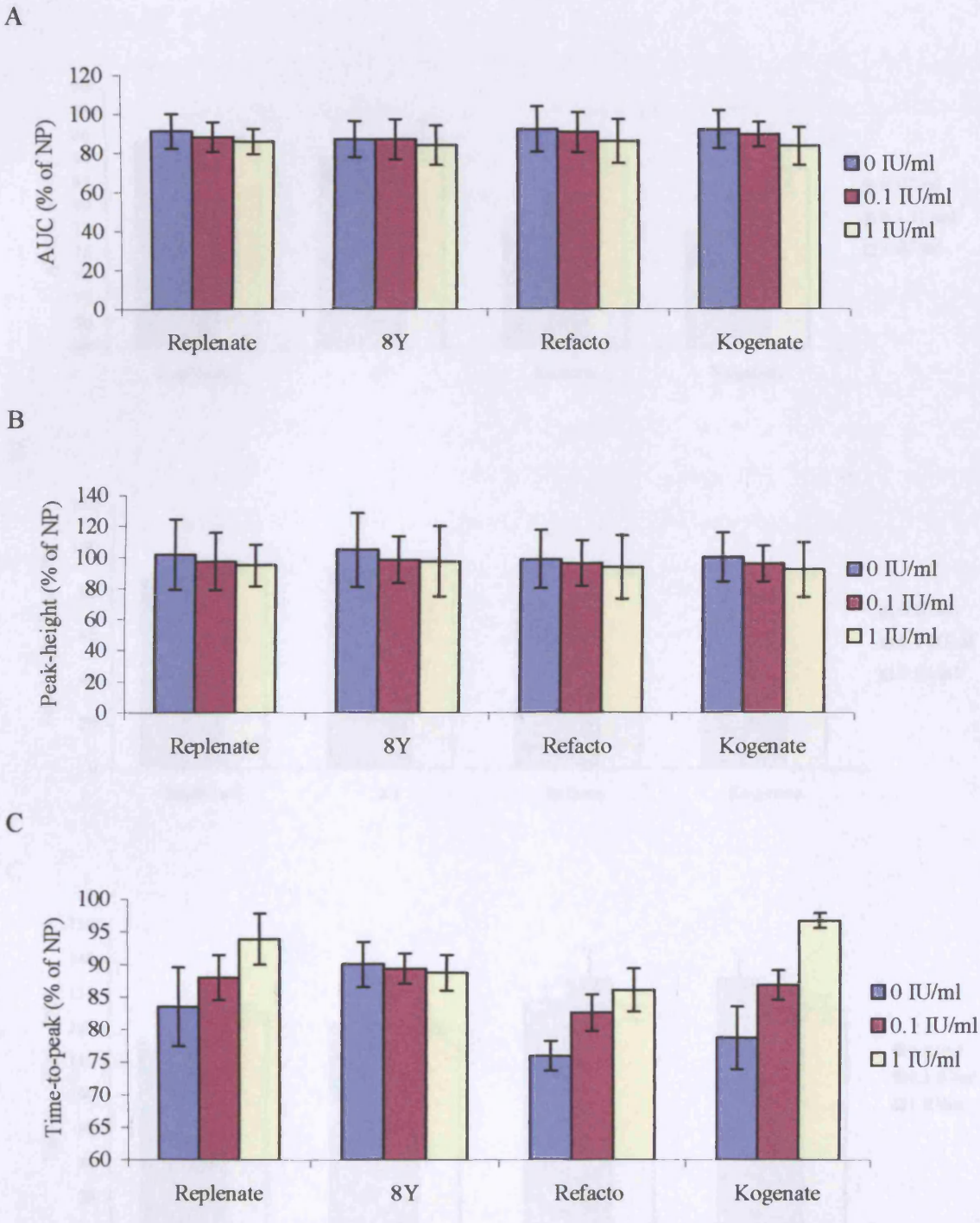
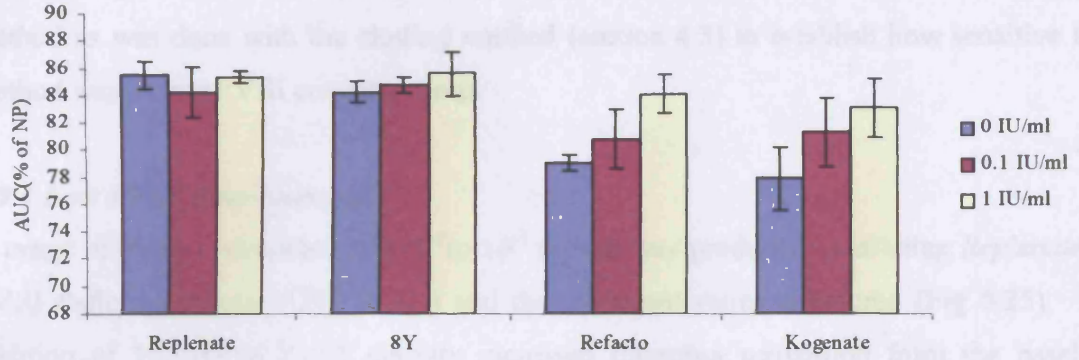
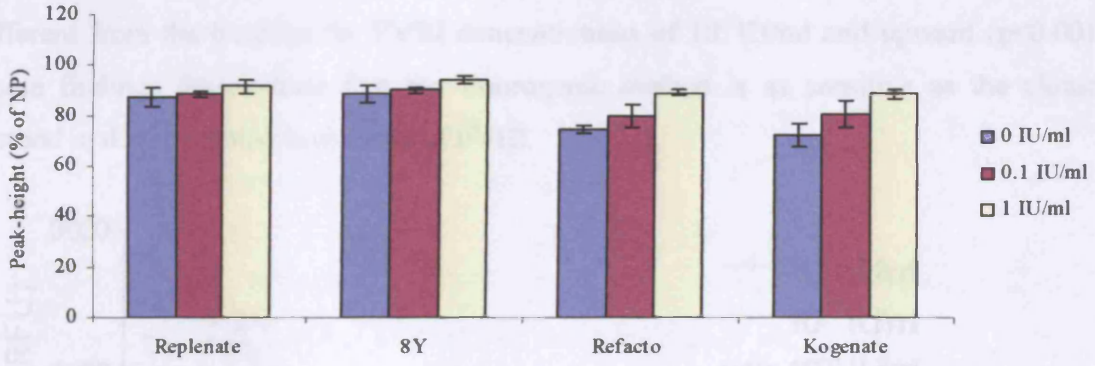


Fig 5.23. Thrombin generated with FVIII concentrates (1 IU/ml) with increasing concentration of VWF(1st IS)(0, 0.1 and 1 IU/ml). A-AUC B-Peak-height C-Time-to-peak.

A



B



C

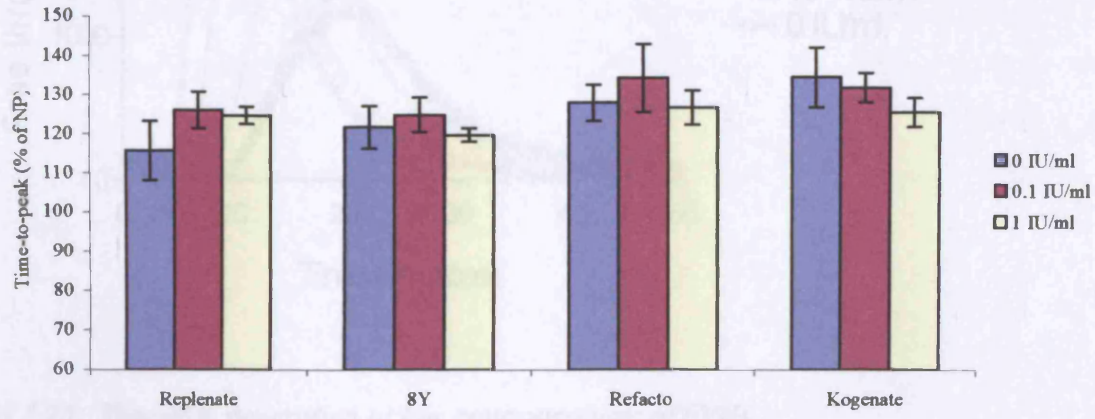


Fig 5.24 Thrombin generation by FVIII concentrates (0.03 IU/ml) with increasing concentrations of VWF (1st IS)(0, 0.1 and 1 IU/ml). A-AUC B-Peak-height C-Time-to-peak.

5.9 Low FVIII concentrations

Thrombin generation was assessed at low concentrations of FVIII with the fluorogenic method as was done with the clotting method (section 4.3) to establish how sensitive this method was to low FVIII concentrations.

5.9.1 Low FVIII dose-response

A range of FVIII concentrations ($10^{-5}$to 10^{-1} IU/ml) was produced by diluting *Replenate* in FVIII deficient plasma (HP1 91hrs) and thrombin generation measured (Fig 5.25). An addition of 10^{-4} IU/ml FVIII slightly increased thrombin generation from the baseline (Table 5.10), this increase however was not significant. Further increases in FVIII concentration increased both the AUC and peak-height and these were significantly different from the baseline for FVIII concentrations of 10^{-3}IU/ml and upward ($p<0.001$). These findings demonstrate that the fluorogenic method is as sensitive as the clotting method in distinguishing low levels of FVIII.

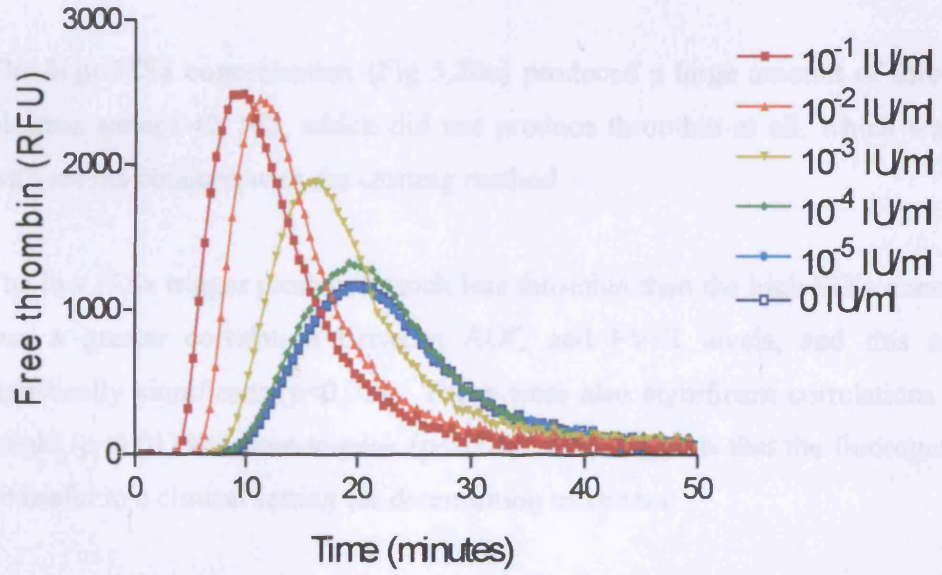


Fig 5.25. Thrombin generation at low concentrations of FVIII.

*Table 5.10. AUC and peak height of low levels of FVIII in the fluorogenic assay with a high FIXa concentration. * $p < 0.001$*

	10^{-1}IU/ml	10^{-2}IU/ml	10^{-3}IU/ml	10^{-4}IU/ml	10^{-5}IU/ml	0 IU/ml
AUC RFU.min	28130±946*	28080±721*	25827±1371*	21400±2063	19634±1377	19593±985
Peak (RFU)	2532±111*	2480±143*	1936±98*	1349±167	1202±87	1199±80

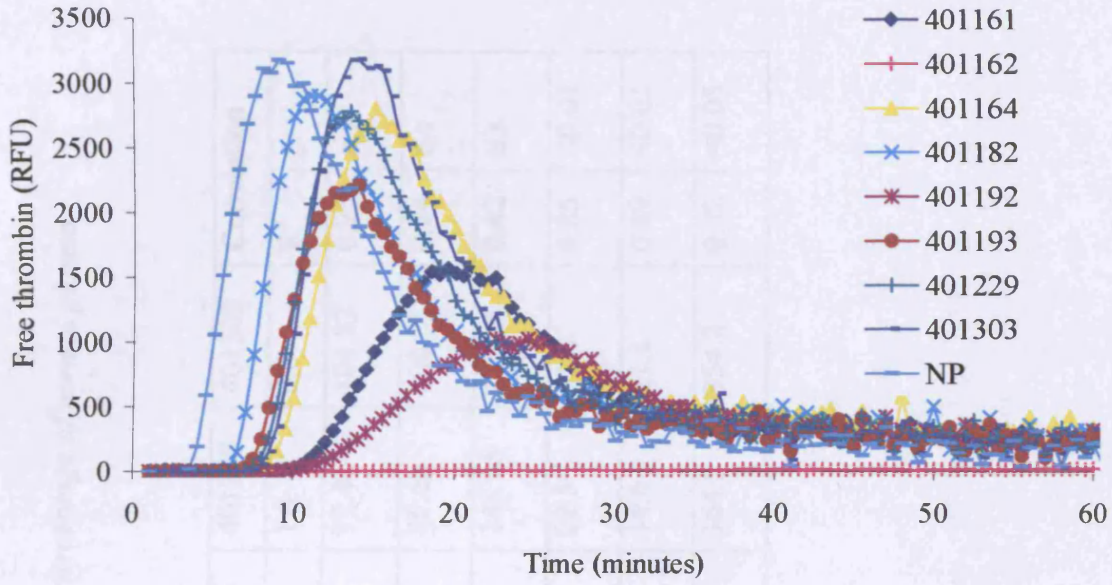
5.9.2 Prophylaxis samples

The prophylaxis samples (section 4.2.2) were also measured by the fluorogenic method using both high and low concentrations of FIXa and PL.

The high FIXa concentration (Fig 5.26a) produced a large amount of thrombin in all the plasmas except 401162, which did not produce thrombin at all, which was in agreement with results obtained with the clotting method.

The low FIXa trigger produced much less thrombin than the high FIXa concentration, there was a greater correlation between AUC and FVIII levels, and this association was statistically significant ($p<0.01$). There were also significant correlations between peak-height ($p<0.01$) and time-to-peak ($p<0.05$). This suggests that the fluorogenic assay could be useful in a clinical setting for determining treatment.

A



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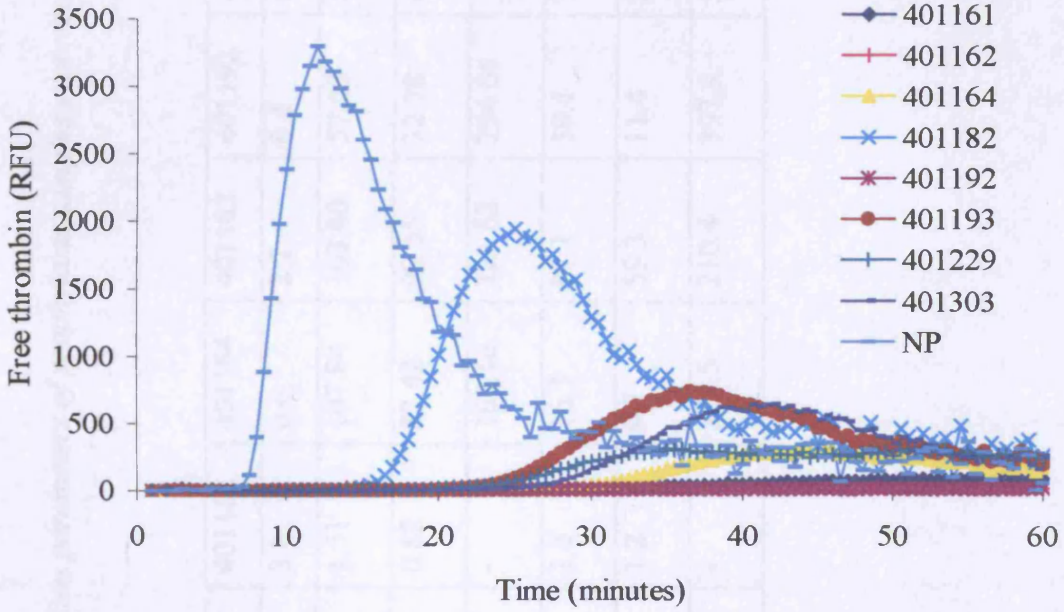

Fig 5.26. Thrombin generation on prophylaxis samples. A high IXa(5nM) B low IXa (0.08nM) .

Table 5.11 Thrombin generation parameters of prophylaxis samples expressed as a percentage of normal plasma.

Sample ID		401161	401162	401164	401182	401192	401193	401229	401303	Correlation	
FVIII IU/dl		0.4	3.0	0.6	2.7	0.4	0.6	1.0	0.8	R^2	p
High IXa (% of NP)	AUC	70.29	1.51	107.84	103.80	57.09	80.89	97.41	104.82	0.25	n.s
	Peak	50.62	0.82	87.42	90.55	32.78	68.93	86.68	100.35	0.24	n.s
	Time	218.92	-	162.16	121.62	254.05	151.35	145.95	154.05	0.42	n.s
Low IXa (% of NP)	Auc	5.1	1.6	16.3	81.1	19.1	37.3	22.5	31.4	0.85	<0.01
	Peak	2.9	1.2	9.7	59.3	11.4	22.6	14.6	21.1	0.89	<0.01
	Time	458.3	-	387.5	210.4	397.9	316.7	364.5	354.2	0.72	<0.05

5.10 Discussion

The results presented in this chapter demonstrate the reliability of the fluorogenic method and that results are comparable to the clotting based method. However, there are differences between the methods in the presence or absence of fibrinogen. In defibrinated plasma a larger AUC, but smaller peak-height is observed in the fluorogenic method, than in the clotting based method. These findings have also been observed by others in the fluorogenic system (Hemker *et al*, 2003) and with subsampling chromogenic methods (Kumar *et al*, 1994;de Bosch *et al*, 2002). However, in their study defibrinated plasma in the fluorogenic assay did not return to the baseline as was observed in Fig 5.4, instead the thrombin level plateaued at approximately half peak-height. The differences in results between the two different fluorogenic methods may be due to the different amount of plasma in the system, in the method used by Hemker *et al* plasma consititutes two-thirds of the reaction mixture, whereas in our method plasma only makes up one-third of the reaction mixture. In a chromogenic subsampling TGT, previous work has shown that in fibrinogen depleted plasma, thrombin generation onset is quicker than that of untreated plasma (Kumar *et al*, 1994;de Bosch *et al*, 2002). This was not found in our subsampling method, but may be due to the differences in subsampling into a chromogenic substrate, as opposed to fibrinogen.

Another experiment was performed by treating plasma with the peptide gly-pro-arg-pro (GPRP), this peptide prevents fibrin polymerisation by binding to the A and B chains of fibrin (Laudano & Doolittle, 1980), thus causing a conformational change (Pratt *et al*, 1997). Plasma treated with GPRP behaved in the same way as defibrinated plasma, which suggests that the increase in thrombin generation was due to the absence of polymerised fibrin. It has been shown that thrombin contained within a clot is able to activate FV, FVIII (Kumar *et al*, 1994), and FXI (von dem Borne *et al*, 1995). Also fibrin alone can activate platelets (Kumar *et al*, 1994), this is mediated through the GPIb receptor on platelets (Béguin *et al*, 1999).

The dose-response to FVIII was measured in the fluorogenic system for both concentrations of the FIXa trigger. The same findings were found as for the clotting system at the high

FIXa concentration i.e. large amounts of thrombin are observed even at very low concentrations of FVIII, peak-height and AUC were similar to normal plasma which contained 0.87 IU/ml FVIII. However, the deficient plasma generated a larger amount of thrombin, AUC 60% of normal plasma, compared to 35% in the clotting system. Thrombin generation could be abolished in deficient plasma by treatment with a polyclonal rabbit anti-FVIII antibody. This would suggest that the fluorogenic TGT is also sensitive to very low levels of FVIII. With a lower concentration of FIXa as the trigger, peak-height and AUC increased with increasing FVIII, while the time-to-peak height decreased. These results mirror those observed in the clotting system.

The thrombin generation profiles were compared for both concentrations of FIXa and also 5pM TF. It has been suggested by several different groups that this concentration of TF may be a closer approximation to the *in vivo* situation as at this concentration of TF, FVIII, FIX and FXI are required for normal haemostasis (von dem Borne *et al*, 1995;Keularts *et al*, 2001a). In general 5pM TF generated similar thrombin generation profiles to that of the low FIXa concentration, however, there was a large difference in the amount of thrombin generated at 0.01 IU/ml. At this concentration the peak and AUC were much smaller than that caused by the low FIXa concentration. This was an interesting finding as this level of FVIII determines the classification of severe haemophilia, and our results suggest that more thrombin is generated if triggered by the intrinsic pathway (FIXa) versus the extrinsic (TF).

The range of FVIII concentrates were compared at a concentration of 0.3 IU/ml for potencies obtained by both the one-stage APTT and chromogenic assay. The product *Kogenate* –SF, which was manufactured without albumin, showed consistently slower thrombin generation for potencies obtained with both methods. Overall, time-to-peak was most similar if potencies had been established by the chromogenic method, this would suggest that this assay is able to give a better reflection on the haemostatic effectiveness of concentrates. This is interesting because the one-stage assay is thought to be more physiological than the chromogenic (Lollar, 2003).

Once it had been established that the new TGT system behaved in the same way as the clotting method, unactivated platelets were added to the system as the source of PL. Platelets were added to determine if there were any differences between the concentrates in ability to generate thrombin in a system that more closely resembled the *in vivo* situation. Haemophilic platelets could not be obtained, therefore platelets were obtained from normal donors and the endogenous plasma containing FVIII removed. The removal of plasma from platelets was not straightforward as it was important to remove all FVIII without activating platelets. A combined procedure of discontinuous albumin gradients followed by gel-filtration was effective in removing FVIII from platelets, whilst leaving them unactivated. The absence of FVIII was demonstrated by our findings with a FVIII ELISA. We also demonstrated that the platelets were unactivated by gel filtration as shown by the absence of FXa generation by unactivated platelets. However, treatment of the platelets with calcium ionophore, which causes the inner leaflet procoagulant phospholipids to be exposed (Bevers *et al*, 1982), showed that when the platelets were activated they were able to take part fully in PL dependent reactions. The fluorogenic assay is comparable to the clotting method, although there is a longer duration until the peak is obtained. This is most likely due to the decreased availability of thrombin due to thrombin being sequestered by the substrate, and therefore unable to partake in feedback activation. A slower rate of thrombin generation has also been observed with the continuous chromogenic method.

The addition of platelets to the TGT was to make the test more physiological, Beguin and Kumar had demonstrated the important link between thrombin, fibrin, VWF and platelets (Béguin & Kumar, 1997). It was thought that the concentrates may behave differently due to the different levels of VWF. However, no difference was observed when four different concentrates with differing VWF concentrations were compared. This would suggest that assay discrepancies are not due to the differing purities of the concentrates. All FVIII concentrates tested at 0.3 IU/ml generated thrombin more rapidly than normal plasma (0.86 IU/ml), this is in contrast to the results with PL in which thrombin was generated after a slightly longer delay as would be expected with a lower FVIII concentration. However, when the reaction was triggered with 5pM TF the FVIII concentrates did not generate

thrombin more rapidly than normal plasma. In addition there were no differences between the concentrates when trigged with TF.

To further study the effect of VWF, FVIII concentrates were tested in a commercial FVIII deficient plasma which was also deficient in VWF. This demonstrated that the lack of VWF had an effect on lag-time; and was confirmed by increasing VWF concentration, which increased lag-time for some of the concentrates. *Refacto* and *Recombinate* had shorter lag-times than *8Y*, which contains VWF in the ratio 1:4 (IU/ml). Addition of VWF to the deficient plasma minimised differences between the recombinant products and *8Y*. *Kogenate*, however had a significantly longer time-to-peak with a normal VWF content than *8Y*. This was in agreement with other TGT experiments in which *Kogenate* was consistently slower to generate thrombin, although this was often not a significant difference. The studies do highlight, though the importance of normal VWF content, and discrepancies may occur if potencies are assessed in VWF deficient plasmas (Barrowcliffe *et al*, 1993b). This confirms previous studies which have shown the importance of VWF in the deficient plasma to minimise discrepancies.

5.11 Summary

The fluorogenic test has been demonstrated to be reproducible and comparable to that of the clotting method. The results presented in this chapter have shown that the method is sensitive to FVIII concentrations as low as 0.001 IU/ml

Potencies as measured by one-stage APTT assay gave more variable lag-times than the chromogenic potencies, suggesting that chromogenic assay gives truer values. VWF was found to influence the lag-time. However, no effect was observed with IP products when assayed in plasma with normal VWF content. This suggests that *in vivo* the haemostatic effects will be the same for all concentrates. In addition when platelets were included in the assay no differences were observed between products of different purity.

CHAPTER 6
GENERAL DISCUSSION AND CONCLUSION

Issues in the measurement of FVIII have been a long standing problem, with discrepancies between assay methods and with the type of standard used. This project has not aimed to study the effect of which standard is used, but rather to use a different approach to try and understand the discrepancies between different FVIII concentrates. Previous studies as detailed in section 1.2.7 have demonstrated that discrepancies between methods can be overcome with the use of the correct standard i.e. like vs like (Barrowcliffe, 1993b). The type of deficient plasma used is also a crucial component and must contain VWF. In addition, other work has shown that more physiological forms of PL can also eliminate discrepancies between one-stage and chromogenic assays in potency measurement of recombinant products in particular *Refacto*.

Thrombin generation was measured for a range of different FVIII concentrates in both a clotting based version and in a fluorogenic adaptation of the assay. In the clotting based assay, *Refacto* was found to generate thrombin more rapidly than the other concentrates at 1 IU/ml. However, *Refacto* was not found to generate thrombin more rapidly than the other concentrates at other concentrations in the clotting system. In addition, in the fluorogenic system *Refacto* was not found to generate thrombin more rapidly than other concentrates. Together these findings suggest that the presence or absence of the B domain does not affect the way in which thrombin is generated.

For all FVIII concentrates used throughout these studies, potency had been established by chromogenic assay. It was confirmed by TGT that the chromogenic assay does give a reliable measure of potency between the concentrates. A direct comparison of thrombin generation following potency measurement by both one-stage and chromogenic assay demonstrated lag-times more closely associated together when potency was assessed by chromogenic. There was no significant difference in time-to-peak between *Refacto*,

Recombinate, *Kogenate*, *Replenate*, *Hemofil-M*, *Octavi* or *8Y*. These results suggest that the chromogenic assay is more accurate in determining haemostatic potential *in vivo* than the one-stage for FVIII concentrates. It is unclear if the chromogenic assay would also be more accurate for plasma samples. The results suggest that all FVIII concentrate potencies should be determined by chromogenic assay. In addition, *Refacto* was found to have the same thrombin generation profiles with the chromogenic potency, suggesting that *Refacto* can be accurately assayed alongside other concentrates.

The FXa generating assay demonstrated large differences between the concentrates in time and quantity of FXa generated. However, these large differences resolved upon thrombin activation, so that, although there were differences between concentrates these were small. This matches findings in the TGT, in that any differences between concentrates are small and unlikely to result in different haemostatic responses *in vivo*. In unactivated concentrates the differences were due largely to the VWF content, with those containing most VWF generating least FXa and more slowly. However, there were still some differences between concentrates when the same amount of VWF was added, suggesting possible differences in the FVIII:VWF complex, or maybe there are slight variations in the ratio of FVIII:VWF, which could be accounted for because of variation in potency assignment.

VWF was shown to be an important constituent of thrombin generation when experiments were performed with and without VWF. In the absence of VWF, thrombin generation was quicker, although there was no difference in the amount of thrombin generated overall. Of the concentrates tested, *8Y* was unaffected by the addition of VWF to VWF deficient plasma. This is most likely due to the presence of a large amount of VWF in this concentrate. The most rapid thrombin generation occurred in the absence of VWF, which mirrors the results of the FXa generation assay. In the absence of VWF, thrombin/FXa is generated more rapidly as FVIII/VWF complex does not need to dissociate to allow thrombin generation. The VWF/FVIII complex dissociates by thrombin cleavage at Arg 1689 (Nogami *et al*, 2000) thereby allowing PL to bind to the FVIII molecule, then thrombin can be generated unhindered. In the absence of VWF, PL can bind to FVIII

without competition. This is presumably a quicker reaction and therefore in the absence of VWF, thrombin or FXa generation will be more rapid. It can therefore be seen that if a FVIII-VWF deficient plasma is used in the one-stage or chromogenic assay then different lag-times will occur in thrombin or FXa generation, resulting in an inaccurate potency determination. It is therefore necessary to equalise VWF levels by using a FVIII deficient plasma which contains normal VWF levels, and this has been reported on several occasions (Barrowcliffe *et al*, 1993b;Mazurier *et al*, 1990).

It was thought that platelets may affect the thrombin generation profile of FVIII concentrates due to the interaction of VWF with platelets. Beguin et al have demonstrated that VWF is important in platelet activation. Reduced thrombin generation was observed in PRP without VWF, which was greater than blocking platelet VWF receptors GPIb and $\alpha_{IIb}\beta_3$ (Béguin & Keularts, 1999). However we found no significant differences in thrombin generation between concentrates containing different amounts of VWF, suggesting that platelets are not necessary for thrombin generation of FVIII concentrates. Presumably because the FVIII deficient plasma supplied sufficient VWF to equalise the different VWF levels between the concentrates.

Kogenate seemed to consistently generate thrombin more slowly than other concentrates, although this was not always a significant finding. This may suggest that less FVIII is present, which would suggest an overestimation of potency by chromogenic assay. However *Kogenate* was assayed against the 6th IS, which is itself a full-length recombinant product, suggesting that potency determination is correct and that the slower generation of thrombin is due to functional differences in this concentrate.

The generation of large amounts of thrombin with very low FVIII concentrations was an unexpected finding as the perceived wisdom is that haemophilic plasma can only generate minimal thrombin. A more important parameter of thrombin generation especially at low FVIII concentrations may be the rate at which thrombin is produced and not the overall amount. A decrease in the rate of thrombin formation may led to a weaker clot which may then be more prone to re-bleeding.

FVIIIa binds to FIXa on PL surfaces to form the tenase complex. These binding sites occur in the A2 and A3 domains of FVIII (Mertens *et al*, 1999). FIX is present in plasma at concentrations of 90nM (Osterud *et al*, 1978) which is a 90 fold excess compared to FVIII (1nM). However, it is unlikely that all FIX is activated during haemostasis. Lawson et al reported normal thrombin generation with one percent of FIX activated (Lawson *et al*, 1994), which would correspond to an approximate 1:1 ratio, which assuming that all FVIII is activated corresponds to the 1:1 stoichometry. Lawson et al found that full activation of the cofactors FVIIIa and FVa were required for maximum thrombin generation. In the TGT used in these studies a high FIXa concentration would correspond to a ratio of 1 FVIII:16.7 FIXa at 1 IU/ml, for the low concentrations of FIXa used this is equivalent to a ratio of 1FVIII:0.26 FIXa at 1 IU/ml FVIII. At low FVIII levels FIXa would be in a much greater excess for example at 0.01 IU/ml this would correspond to 1:1670 for high FIXa concentration, and 1:26 at low FIXa concentration. It has been found in an epidemiology study of normal population that FVIII levels are correlated with FIX (Lowe *et al*, 1997).

It is currently unclear how FIXa increases thrombin generation at low FVIII levels, however, two mechanisms are likely. Firstly, increasing the concentration of FIXa would increase the amount of FVIII in complex, the Kd of this association is 0.5nM (Jesty, 1990;Rawala-Sheikh *et al*, 1990). Secondly, at higher concentrations of FIXa inactivation of FVIIIa through A2 subunit dissociation is reduced (Fay *et al*, 1996).

FIXa was also found to protect FVIII from a polyclonal antibody. This could in complex with PL and Ca^{2+}, protect infused FVIII from inactivation by circulating antibodies in an inhibitor patient. It is also possible that FIXa could be used alongside FVIII to increase the effectiveness of FVIII in non-inhibitor patients and thereby allowing a reduced consumption of FVIII.

The main bar to the use of FIXa in a therapeutic setting is the highly thrombogenic nature of FIXa. Increased levels of FIX have been associated with increased risk of venous thrombosis in case-control studies (Lowe *et al*, 2000), this study did however find

associations with other coagulation components. FIXa as a contaminant of FIX concentrates has been found to cause thrombosis in animal models (Gray *et al*, 1995;Kusch *et al*, 1998). However, as those with haemophilia have a bleeding tendency they may not suffer from the adverse effects of FIXa.

Although estimates have been made of the amount of FIXa generated *in vivo* these have all been based on *in vitro* or computational models. In addition, it is unclear how much FIXa is generated in *in vitro* experiments with 5pM TF which is the preferred trigger. Knowing this would allow greater understanding of the role of FIXa in thrombin generation.

In conclusion, the TGT has shown that there are no differences between concentrates when measured by the chromogenic assay, this suggests that there are no differences *in vivo*. The concentration of FIXa used to trigger TGT is crucial and can promote thrombin generation with low FVIII levels. In addition, FIXa in a complex with FVIII, PL and Ca^{2+} is able to protect FVIII from antibodies, which could be useful therapeutically.

The fluorogenic TGT is a version of the TGT which could potentially be used on a routine basis. The fluorogenic method was found to be comparable to the clotting method; the main difference is in the time-to-peak which is delayed in the fluorogenic, which implies that the substrate interferes with thrombin generation. The reproducibility of the fluorogenic assay was found to be high with inter-assay variability of 6.4-8.8% in time-to-peak. The assay also has an increased level of sensitivity (0.001 IU/ml) over the chromogenic and one-stage assays. A further advantage is the minimal amount of handling required of the sample and also the small volume of the sample (40µl) per measurement. This method can also be carried out both without and with platelets which would allow testing to be as close as possible to the *in vivo* situation. The fluorogenic TGT is an easy assay to use and apart from the cost of the fluroimeter all other reagents including the substrate are inexpensive. This assay therefore has the potential to become a routine assay.

Future work

The TGT has shown great promise for the detection of very low levels of FVIII. More work would need to be done to see if thrombin generation is a good parameter for measuring treatment. It may be an important tool for evaluating outcome of gene therapy trials. An increase in base-line thrombin generation after gene-therapy would demonstrate an increased FVIII level which is beyond the sensitivity of current assays. Another interesting use would be to examine a large group of patients at a long duration post-infusion to see if there is a correlation between thrombin generation profile and other clinical measure like joint bleeding and FVIII usage.

Further work needs to be performed to examine the protective effect of FIXa/PL/Ca^{2+} on FVIII as there is potential for a therapeutic product, if thrombosis is not found to occur in *in vivo* models.

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McIntosh JH, OwensD, Lee CA, Raut S, Barrowcliffe TW. (2003)A modified thrombin generation test for the measurement of factor VIII concentrates. *Journal of Thrombosis and Haemostasis*, **1**, 1005-1011

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ORIGINAL ARTICLE

A modified thrombin generation test for the measurement of factor VIII concentrates

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